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Editorial

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Some Problems of Vaccination Campaigns in Developing Countries

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Vaccination offers the most cost-effective approach to prevent and control infectious diseases in the history of mankind.[1] The English physician Edward Jenner introduced smallpox vaccine in 1798. It is the first successful vaccine to be developed. Since then a series of vaccines have been developed and come into use; for example, the influenza vaccine, the hepatitis B vaccine, and the polio vaccine. The World Health Organization (WHO) reports that licensed vaccines are currently available to successfully combat against twenty-five infections.[2]

Recently the potential epidemics of two infectious diseases have stepped into the spotlight: diphtheria resurgence in Denmark[3] and swine flu (H1N1) outbreak in Ukraine.[4] Danish authorities announced in this January that unvaccinated refugees have brought deadly diphtheria into the country after 20-year absence. For other European countries, it was reported that asylum seeker have also been found to carry tuberculosis and malaria. Flu activity most commonly peaks in the northern hemisphere between December and February. In Ukraine, the H1N1 influenza virus has killed more than 50 people in this January so far, and moreover, the infection cases are growing, and many young people have fallen ill. The virus is approaching, or might have reached, epidemic levels in parts of the country. Swine flu (H1N1) has caused deaths in Russia and other former Soviet republics as well such as Armenia and Kazakhstan.

That vaccine-preventable diseases are rampant in developing countries may be induced by the following factors:

- Many people cannot afford to finance vaccine research or purchase the vaccines such that they are still not benefiting from vaccination.
- Both doctors and the general population lack a culture of vaccination in developing countries. Before or during an epidemic, the number of physicians suggesting their patients to be vaccinated is extremely low, and the percentage of those who have actually received their immunization is practically non-existent. More seriously, people are used to not seeking treatment until the disease has progressed to a difficult-to-treat stage.
- Anti-vaccination rumors contribute to the seriousness of preventable disease outbreak. In Eastern Africa and South and West Asia some individuals and media organizations spread rumors that oral polio vaccine was contaminated with Human Immunodeficiency Virus (HIV) and anti-fertility drugs. Another rumor says that vaccines cause child autism. Afghanistan and Pakistan account for the vast majority of polio cases globally and are the only two countries where it remains endemic. Some militant groups prohibit polio vaccinations and have attacked health workers. They claim that the polio vaccination drive is a front for espionage or a conspiracy to sterilize Muslims. Some Pakistani parents are against vaccination as well and they believe they are part of a Western plot to sterilize children.[5]
- Corruption in the health sector can mean the difference between life and death. Poor people are usually worst affected by graft. Corruption costs lives when fake or adulterated medications are sold to health services. This can lead to the public distrust in health system. As to fake and/or out-of-date vaccine, in 2009 more than 1,600 people in Guangxi Zhuang Autonomous Region, China have been injected with fake rabies vaccine and at least one boy died as a result[6]; hundreds of children from central Chinese province of Henan suffered serious health problems after being given out-of-date vaccines in 2014.[7]

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What is so inspiring is that the Global Alliance for Vaccines and Immunization (GAVI) Alliance, a public-private global health partnership established in 2000 has been committing to increasing access to immunization in poor countries. GAVI has raised money for vaccines to save the lives of millions of children every year; United Nations Children's Fund (UNICEF) is helping countries with the training of health workers and helping governments set up the systems around communicating with parents and caretakers so that they understand why it is important to immunize their kids, and what they can expect from these vaccines; in addition, The WHO, UNICEF, The World Bank and other agencies and officials are stopping vaccine rumors by combating fear with knowledge and evidence, not with coercion.

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Current Status of Human Immunodeficiency Virus Vaccines

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Highly Active Antiretroviral Therapies (HAARTs) have been developed to treat HIV+ individuals, increasing the quality and quantity of life of many HIV+ patients. Despite these effective strategies, human immunodeficiency virus infection and acquired immune deficiency syndrome (HIV/AIDS) epidemic continues to uphold globally with 39 million infected individuals.[1] However, the HIV retrovirus integrates into the (cluster of differentiation 4) CD4+ cells' genomes where it can persist for years in a latent stage forming HIV reservoirs throughout the body.[2] Due to these reservoirs, infected individuals need to be under treatment for the rest of their lives, as HAARTs cannot fully cure HIV.[3,4] As a result, preventive strategies have now been introduced in the plan to completely eradicate the HIV/AIDs endemic.[5] Effective preventative strategies that decrease bodily fluids transmissions like the usage of condoms, sterile needles, abstinence, monogamy between uninfected individuals, and voluntary testing have been developed. The development of vaccines as preventative and treatment strategies against HIV has been proposed to aid in the eradication of this disease.[5,6] Typically, vaccines have been successful in the disappearance of past endemics such as polio and small pox. Vaccine development for HIV began in the 1980's.[1] Two types of vaccines have been proposed: those as protection against acquisition of HIV-1 and those as treatment to cure HIV in conjunction with HAART.[1,5]

Vaccines with the purpose of reducing HIV acquisition are controversial due to its necessity to compete with the broad genetic diversity of HIV and overcome multiple transmission modalities.[7] Nonetheless, a few have gone through phase 3 and phase 2b clinical trials. Examples of these case trials include VAX 004, VAX 003, Step, Phambili, RV144, and HVTN 505. While efficacy in VAX 004, VAX 003, Step, and Phambili clinical studies were not achieved in regards to finding significant differences in HIV acquisition for both the vaccinated and placebo groups, another type of vaccine has had a more successful outcome.[8,9] The RV144 study showed the potential for an HIV/AIDS vaccine to prevent infectivity of HIV.[10] This study used a vaccine combining the bivalent (B/E) gp120 vaccine used in VAX 003 with an ALVAC vector prime. The results showed a 60.5% vaccine efficacy at 1 year and 31.2% vaccine efficacy at 3.5 years with ALVAC-HIV (vCP1521) (0, 1, 3, 6 months) followed by protein boosts with alum adjuvant, AIDS-VAX1 clades B/E gp120 (3, 6 months). Further studies using these vaccines have suggested that the V2 region of HIV-1 is a target site of protective antibodies associated with vaccine efficacy of the RV144 regimen.[11] Due to a reduced efficacy over time, there is a potential for waning immunity in respect to HIV acquisition over a period time after vaccination needs to be resolved. Currently, studies are being conducted to improve efficacy of this vaccine through the use of different adjuvants, MF591 and ASO1B, and improvement of B- and T-cell priming by the introduction of immunogenic vector platforms. Studies using vaccines with a replication-incompetent Ad26 vector in combination with MVA/trimeric gp140/ASO1B adjuvant have shown protection from mucosal challenge. In a non-human primate study, a correlation among envelope-specific non-neutralizing binding antibodies with protection against acquisition have been demonstrated.[11]

Vaccines as therapeutic treatment for HIV are being developed to eradicate HIV res-

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ervoirs, HARTs major obstacle.[12] Reservoirs persist due to the invisibility of latent provirus provided by: (1) dormant CD4+ T-cells against the body's immune system, (2) HARTS inefficient targeting of activated CD4+ T-cells, and/or (3) non-induction of immune response due to low levels of residual virus production. In order to combat these obstacles, a "shock and kill" strategy has been developed. This strategy consists of therapeutic vaccines introducing HIV-specific T-cells capable of killing the reactivated infected cells producing HIV antigens. A phase I/II, open-label, single-arm clinical trial has been conducted to evaluate a dendritic cell (DC) based HIV-1 vaccine loaded with autologous HIV-1-infected apoptotic cells.[13,14] Results showed the vaccine was safe and induced T-cell activation. However, it did not prevent viral rebound during treatment interruption. Four of 10 participants had an increase in the HIV-1 ribonucleic acid (RNA) load in plasma following vaccination, despite continuous antiretroviral therapy (ART). Evidence of cytolysis in HIV-1-infected cells was also present.[14] Other studies evaluating TAT therapeutic vaccines have shown TAT vaccination is safe and immunogenic. A non-blinded with no placebo controls phase 2 study gave evidence for restoration of CD4+ and CD8+ T-cell numbers and functional central memory T-cell subsets of B and natural killer (NK) cell number, and a reduction of immune activation in HAART-treated participants. Furthermore, TAT immunization induced a statistically significant reduction of blood HIV-1 deoxyribonucleic acid (DNA) load that persisted for up to three years post-vaccination.[11]

To conclude, antibody-mediated preventative HIV vaccines will in the future provide a great strategy in the eradication of the HIV epidemic pending further developments of neutralizing antibodies. VRCO1, a human monoclonal antibody targeting the HIV-1 CD4 binding site, has demonstrated protection in animal studies, and has acceptable human safety. HVTN 703 will investigate the effectiveness of VRC01 and the level of neutralizing activity required in reducing HIV acquisition. As for therapeutic vaccines, further studies need to be conducted using TAT based vaccines in order to evaluate its efficacy in reducing HIV-1 DNA.

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Cell Derived Virus-Like Particles (VLP) in Future Vaccine Development

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Traditionally, viral vaccines have been based on inactivated or live attenuated viruses. While in general, they are highly effective, in some cases they fail to provide adequate immunogenicity, safety or can even cause adverse events. In the case of live attenuated vaccines, achieving a stable optimally attenuated virus is often difficult and there is the potential for reversion. Transmission to the immunocompromised individuals is an additional concern. Inactivated vaccines run the risk of inducing enhanced disease. Single proteins, including single protein nano-particle vaccine attempts have not been successful to date for human use. Various other ways of making vaccines have also been attempted by engineering the virus.

Virus-like particles (VLP) show much promise as future vaccines. VLP vaccines such as HPV are already available commercially. These VLPs are safe because they are devoid of any viral genetic material and therefore not infectious.

The VLP technology using the expression of one or more viral structural proteins in cells from cDNA results in spontaneous assembly of particles that resembles the real virus morphologically and immunologically. Larger the number of viral protein particles better the immune response expected to be. However, for the vaccines to be cost-effective, the number of proteins have to be limited; here the improved immune response has to be taken care of by adjuvants.

We used a mammalian cell-derived VLP technology to generate VLPs in a short time and to develop vaccines on the fast tract. Fundamentally, the technology can be used to generate VLPs for any virus. We have produced Nipah virus VLPs (NiV VLPs) and shown that the adjuvanted NiV VLPs protects in the hamster model with single inoculation. We have produced respiratory syncytial virus (RSV) VLPs and shown that the adjuvanted RSV VLPs protects in both cotton rat and mice models the lower and upper respiratory tracts. Adjuvanted RSV fVLPs showed potent neutralizing antibody response and protection in mice. We have shown that we can develop VLPs in a short time. Zika VLP proteins were assembled together and VLPs were made in less than 2 months. The technology has the potential to handle unexpected, uncontrolled outbreaks quickly.

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Recent Advances in Adenovirus-Vectored Vaccines Development

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Recombinant adenovirus-vectored vaccines based on human adenovirus serotype 5 (HAdV-5) have been extensively studied both pre-clinically and in clinical trials for the past 25 years. Initially, they were considered as the most promising platform for human immunodeficiency virus (HIV) vaccine development. However, HAdV-5-based vaccine did not meet expectations in a large-scale clinical trial called STEP trial.[1] In that trial, the vaccine not only showed lack of efficacy, but also suggested an increased trend for HIV acquisition in individuals with pre-existing HAdV-5 neutralizing antibodies.

Researches have developed vectors based on alternative serotypes of human and non-human adenoviruses in order to overcome challenges with HAd5-based vectors. To date, HAdV-35, HAdV-26 and simian adenoviruses ChAd3, ChAd63 and ChAdOx1 have been tested in several phase 1 clinical trials as candidate vaccine component against *Mycobacterium tuberculosis*, *Plasmodium falciparum*, HIV, Ebola, HCV and influenza virus. These vectors were chosen because most people have little or no immunity to them, and their biological characteristics, such as utilization of primary cellular receptor and elicitation of innate cytokine responses, differ from HAdV-5.

Vaccine Ad35-TBS (or AERAS-402) consists of recombinant replication-defective HAdV-35 vector expressing *M.tb* antigens Ag85A, Ag85B and TB10.4 as a single fusion protein.[2] A phase 1 trial in healthy Bacillus Calmette-Guerin (BCG)-vaccinated adults in South Africa demonstrated that the vaccine is safe and immunogenic.[3] In addition, the vaccine safety and immunogenicity has been reported in healthy adults living in the US.[4] In the study, volunteers were primed with BCG three or six months prior to AERAS-402 boosting. Also, AERAS-402 was safe and immunogenic in healthy infants previously vaccinated with BCG at birth.[5]

Vaccine Ad35.CS.01 is a pre-erythrocytic malaria candidate vaccine. To make the vaccine, the codon optimized nucleotide sequence of *P. Falciparum* circumsporozoite (CS) surface antigen was inserted in the E1 region of a replication deficient HAdV-35 vector. Phase 1 trial demonstrated that the vaccine was well-tolerated and modestly immunogenic in healthy adults living in the US or Sub-Saharan Africa.[6,7]

The development of a vaccine to prevent HIV infection remains a global health priority. Therefore, recombinant HAdV-26 and HAdV-35 with HIV clade A envelope gene inserts were constructed. In a randomized, double-blind, placebo-controlled, multicenter, international clinical trial in the US, Kenya, Rwanda and South Africa both vaccines elicited significant immune responses in all populations. Baseline vector immunity did not have a significant impact on immune responses, and second vaccinations in all regimens significantly boosted EnvA immunity.[8]

Another approach was developed based on designing mosaic antigen using genes from different HIV subtypes responsible for HIV-1 infections worldwide. The antigen was expressed in recombinant replication-defective HAdV-26 vector adenovirus serotype 26-Mosaic -human immunodeficiency virus (Ad26.Mos.HIV). A phase 1/2a study (named APPROACH) in 393 healthy HIV-uninfected adults has been conducted in the US, Rwanda, Uganda, South

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Africa and Thailand. Vaccine regimens contained two prime doses of vector Ad26.Mos.HIV and two boosts of either Ad26.Mos.HIV, MVA-Mosaic and/or different doses of the soluble protein Clade C gp140 adjuvanted with aluminum phosphate. As presented at the 9th IAS Conference on HIV Science (IAS 2017), the results indicated that the "mosaic"-based vaccine regimen appeared to be well-tolerated and elicited HIV-1 antibody responses in 100% participants.

Chimpanzee-origin vectors tested in humans were derived from serotype 63 (ChAd63), and were used to express the pre-erythrocytic malarial antigen ME-TRAP.29.[9] Hundreds of individuals had been immunised in Africa and UK with ChAd63-vectored malaria vaccine.[10,11] Similarly, serotype 3 (ChAd3) vector expressing non-structural proteins from hepatitis C virus (HCV) genotype 1b successfully induced a T-cell response against HCV in healthy volunteers.[12,13] In addition, simian adenovirus vector ChAdOx1 expressing the conserved influenza antigens, nucleoprotein (NP) and matrix protein 1 (M1), was constructed and shown to be safe and immunogenic in adult humans.[14]

As a rapid response to the 2014 Ebola epidemic, adenovirus-vectored vaccines were developed and subsequently tested in phase 1 clinical trials. Those were a replication defective recombinant chimpanzee adenovirus ChAd3-vectored vaccine (cAd3-EBO), encoding the glycoprotein (GP) from Zaire and Sudan species and a replication-defective HAdV-26-vectored vaccine expressing GP from the Zaire Ebola virus (Ad26.ZEBOV). Ad26.ZEBOV was used in a combination with MVA-BN-Filo in a heterologous prime boost vaccination strategy with MVA-BN-Filoas a booster vaccine. Both these vaccines were well tolerated and immunogenic in healthy adults.[15,16]

CONCLUSION

In conclusion, although adenovirus-based vectors have seen their share of setbacks in recent years, they remain to be a valuable tool for vaccination against infectious diseases. Development of novel vectors based on alternative human and non-human serotypes helps to overcome challenges observed with HAdV-5-based constructs.

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Leading Ebola Vaccine Candidates

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The ongoing outbreak of Ebola Virus Disease (EVD) in West Africa is the largest outbreak ever recorded with a total number of 28,602 confirmed, probable, or suspected cases in Guinea, Liberia, and Sierra Leone, including 11,301 reported deaths since December 2013 (as of January 17, 2016).[1] A meeting convened by the World Health Organization (WHO) in September, 2014, concluded that an urgent unmet need exists for efficacy and safety testing of the EVD vaccine candidates and that clinical trials should be expedited. These vaccines could be used both in an outbreak setting and to provide long-term protection in populations at risk of sporadic outbreaks.

A number of vaccines have been evaluated in phase 1 trials including DNA vaccines, virus-like particles and viral vectors,[2] but the two most advanced first-generation Ebola vaccines candidates are the live replicating Vesicular Stomatitis Virus (rVSV) and the replication-defective chimpanzee adenovirus 3 (ChAd3).

rVSV-ZEBOV

The replication-competent recombinant vesicular stomatitis virus (rVSV)-based vaccine expressing the Glycoprotein (GP) of a Zaire strain of Ebola virus (ZEBOV) is among the leading Ebola vaccine candidates and has been rapidly progressed to a phase 3 efficacy trial in Guinea.

A key determinant of VSV pathogenicity is the surface GP which is also the predominant target for immune responses. The rVSV Ebola vaccine (rVSV-ZEBOV) is designed to exploit this by replacing the VSV-GP with a GP from the Zaire Ebola virus (strain Kikwit-95). This chimeric design with switching of GPs attenuates the pathogenicity of the virus while allowing the vaccine virus to replicate using the Ebola GP to attach and enter cells.[3]

The vaccine was developed by the Public Health Agency of Canada, licensed to Bio-Protection Systems (NewLink Genetics, IA, USA), and most recently sublicensed to Merck, which is responsible for ongoing research and development.

The rVSV-ZEBOV vaccine has been assessed in eight phase 1 studies in Europe, Africa, and North America; a large phase 2 study (the PREVAIL study, NCT02344407) in Liberia; and an ongoing phase 3 study in Sierra Leone (the STRIVE study, NCT02378753) and more than 9000 volunteers have received this vaccine so far.

Preliminary results from open-label, dose-escalation phase 1 trials and randomized, double-blind, placebo-controlled phase 1 trials have been reported in April 2015 in *The New England Journal of Medicine*.[4,5] These trials assessed safety, side-effect profiles, and immunogenicity of rVSV-ZEBOV at various doses in healthy adults in Europe, Africa and US. Participants were injected intramuscularly (IM) with doses of vaccine ranging from 300,000 to 50 million Plaque-Forming Units (PFU) or placebo.

The most common adverse events were injection-site pain, myalgia, and fatigue. Transient VSV viremia was detected within 3 days in most participants receiving 3 million PFU or more. Fever was observed in up to 35% of vaccinees. At the Geneva trial site where participants were dosed with 10 million or 50 million PFU of rVSV-ZEBOV, 11/51 participants developed

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arthralgia in the second week after injection.[4] Reducing the dose of rVSV-ZEBOV from 10 million (or greater) to 300,000 PFU improved its early tolerability but lowered antibody (Ab) responses and did not prevent vaccine-induced arthritis, dermatitis, or vasculitis.[5] Thus, although the mechanism of this arthritis remains unclear, it is likely that local VSV replication may play a role. It will be critical to confirm the frequency of these side effects in the large phase 3 efficacy trial being run in Guinea to enable a more informed evaluation of the risk of live rVSV vaccination.

Regarding the induction of specific Abs, the rVSV-ZEBOV vaccine generated GP-binding Abs in all participants at all doses (as few as 300,000 PFU may be sufficient), showing its immunogenicity in humans. In the study undertaken in the US, Ab titers against the Ebola Zaire GP, at day 28, were higher in the group receiving 20 million PFU than in the group receiving 3 million PFU.[6] These data support continued development of the rVSV-ZEBOV Ebola vaccine candidate in general and the selection of a dose of 20 million PFU for phase 2 and 3 trials.

It was noteworthy that despite similar GP-binding Ab titers between groups administered with doses between 3 million and 50 million PFU, higher vaccine doses elicited higher titers of neutralizing Abs to the Ebola Zaire GP. Since the relative roles of neutralizing and GP-binding Abs in protection against Ebola virus disease are unknown, it is difficult to conclude whether higher vaccine doses are required for optimal protection. However, a preliminary comparison of data from Non-Human Primates (NHPs) vaccinated with the same doses (3 million or 20 million PFU) used in the US trials, then subsequently challenged with Ebola-Kikwit strain virus, showed that survivors had significant pre-challenge IgG-Ab responses against Ebola GP, as seen in vaccinated human volunteers.[5]

Further follow up from these studies to determine the durability of Ab responses is awaited. One important unanswered question is whether rVSV induces any cellular immunity and how these immune responses correlate with protection.

The *Ebola ça Suffit* ("Ebola this is enough") phase 3 trial is currently underway in Guinea to assess the efficacy of the rVSV-ZEBOV candidate vaccine for the prevention of EVD. The preliminary report of the Guinea trial, published in August 2015 in *The Lancet*, reported very encouraging results following a planned interim analysis.[7] The trial tested a ring vaccination design, a strategy that was borrowed from successful smallpox eradication efforts in the 1970s; after one patient contracts the disease, close contacts are identified and those who are eligible to receive vaccination and can give consent are vaccinated in the hope of stemming the onward spread of the virus.

The Guinea trial included two arms: one in which adults who had been in contact with someone infected with Ebola and their subsequent contacts were vaccinated shortly after the original patient developed Ebola, and a second in which contacts instead received the vaccine three weeks later (one dose of 20 million PFU, administered IM).

Between April 1, 2015, and July 20, 2015, 7651 people were included in the planned interim analysis. Four-thousand one-hundred and twenty-three people were randomly assigned to immediate vaccination with rVSV-ZEBOV, and 3528 people were randomly assigned to delayed vaccination. In the immediate vaccination group, there were no cases of Ebola virus disease with symptom onset at least 10 days after randomization, whereas in the delayed vaccination group there were 16 cases of EVD. The findings mean that the vaccine provided 100% protection from the virus, though the study's small size means that the vaccine's true protection rate may be slightly lower. The authors of the paper estimate its true effectiveness at between 75% and 100%. No new cases of EVD were diagnosed in vaccinees from the immediate or delayed groups from 6 days post-vaccination. Forty-three serious adverse events were reported; one serious adverse event was judged to be causally related to vaccination (a febrile episode which resolved without sequelae).

This study enables some cautious preliminary conclusions and suggests that rVSV confers protection between 6-21 days after vaccination; how much longer vaccine-induced protection lasts is unknown. Vaccine failures within the first 6 days would suggest that vaccine-induced protection needs at least a week to reach effective levels and raises some doubt on the claim that rVSV could work rapidly and provide post exposure prophylaxis. A non-significant indirect protective effect among unvaccinated individuals was also observed. The magnitude of this effect and whether it was mediated through a reduced viral load and transmission remains to be determined. Lastly, this study offers a unique opportunity to identify vaccine-induced correlates of protection. In phase 1 trials, the Ab titers induced by rVSV vaccination reached titers associated with protection in NHPs only by 28 days post vaccination,[8] implying that the Ab titer required for protection of humans against naturally acquired infection is likely lower than that required for protection of NHPs against controlled challenge.

In the wake of the trial results, the WHO has decided that the rVSV-ZEBOV vaccine will continue to be used in the outbreak in Guinea as part of the clinical trial. A combined phase 2 and phase 3 clinical trial designed to assess the safety of the rVSV-ZEBOV candidate (20 million PFU) is being conducted in Sierra Leone (STRIVE: Sierra Leone Trial to Introduce a Vaccine against Ebola).

rVSV-ZEBOV remains a 'first-generation' vaccine that is not ideal for stockpiling: it must be stored at -80 °C and only protects against a limited number of species of the Ebola virus. Gavi, the vaccine alliance in Geneva, Switzerland, will work with researchers and industry to support the development of second-generation Ebola vaccines that target other Ebola virus species, as well as the closely related Marburg virus, and which do not require storage in expensive, laboratory-grade freezers. Gavi has signed an advance purchase agreement with Merck that gives the drug company $5 million to develop the

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rVSV-ZEBOV-GP Ebola Zaire vaccine. The agreement is based on the understanding that 300,000 doses of the vaccine will be available for clinical trials or emergency use by May 2016 and that the vaccine will be submitted for full licensure by the end of 2017.[9]

ChAd3-ZEBOV

The monovalent, replication-deficient, chimpanzee adenovirus type-3 vector-based Ebola Zaire vaccine (ChAd3-ZEBOV, also known as ChAd3-EBO-Z or cAd3-ZEBOV) encoding the Ebola GP from the Zaire strain has undergone extensive preclinical development and is now among the leading Ebola vaccine candidates. The original clinical-development plan for this Ebola vaccine, primarily for biodefense, included the use of a bivalent vaccine formulation of Zaire and Sudan strains[10] that would use both ChAd3 and Modified Vaccinia virus Ankara (MVA) viral vectors. The ChAd3 vaccine encoding just the Zaire strain appeared to be a potentially advantageous monovalent formulation for outbreak control on the basis of efficacy data in macaques and was thus selected for phase 1 clinical testing.

The ChAd3-ZEBOV was developed by the Vaccine Research Center (VRC) of the National Institute of Allergy and Infectious Diseases (NIAID) in collaboration with Okairos (now a division of GlaxoSmithKline).[11]

The ChAd3-ZEBOV had already been manufactured to clinical grade at the time of the acceleration of the EVD outbreak in early August 2014. These events provided the opportunity to design a rapid clinical development program that could lead to deployment of the vaccine. In September 2014, two phase 1 clinical trials began and have reported preliminary results on the use of a single dose of ChAd3. The dose-escalation clinical trial, called VRC 207, was performed in the US (conducted at the National Institutes of Health – NIH), to determine the safety, side-effect profile, and immunogenicity of the bivalent vaccine that included ChAd3-EBO glycoprotein Zaire and ChAd3-EBO glycoprotein Sudan, in a 1:1 ratio.[10] A total of 20 participants were enrolled and vaccinated with a single dose of vaccine administered IM at a dose of 2×10^{10} particle units (pu) or 2×10^{11} pu. This bivalent vaccine was well tolerated with self-resolving mild to moderate side effects. As expected, the higher dose induced significantly higher magnitude of Abs and T-cells compared with the lower dose, with responses peaking 4 weeks after vaccination. The authors found that Ab titers in individuals vaccinated with the higher dose were within the range associated with protection in NHP models.[11,12]

The second phase 1 clinical trial, called EBL01, began in the UK at the Jenner Institute (University of Oxford). EBL01 was a dose-escalation, open-label study assessing the safety and immunogenicity of the monovalent ChAd3-ZEBOV.[13] Three dose-specific groups of 20 volunteers each were recruited for the trial and were assigned to receive the ChAd3 vaccine as a single IM injection: 1×10^{10} pu, 2.5×10^{10} pu or 5×10^{10} pu.

The safety profile was similar to that observed in the US study, with no vaccine-related serious adverse event at any of the dose levels studied (fever developed in 2 of the 59 participants who were evaluated).

Ab responses were higher at 4 weeks in the high-dose group. However, in contrast to the US study, GP-specific Ab titers were lower than those induced in macaques protected by the same vaccine. At the vaccine doses tested, T-cell responses were detected (more $CD4^+$ than $CD8^+$ T-cell responses according to the secretion of IFN-γ, IL-2, or TNF-α) and peaked at 14 days rather than 28 days as observed in the US study.

Between October 24, 2014, and June 22, 2015, a third phase 1/2a trial evaluating safety and immunogenicity of ChAd3-ZEBOV was performed at the Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland (trial number NCT02289027).[14] In this randomized, double-blind, placebo-controlled, dose-finding trial, participants received a single IM dose of low-dose vaccine (2.5×10^{10} pu), high-dose vaccine (5×10^{10} pu) or placebo.

A sample size of 100 vaccinated participants was calculated to achieve a total of 250 vaccinated participants, taking into account all three concurrent phase 1 trials of the ChAd3-ZEBOV vaccine (Lausanne, Oxford, and Mali). This sample size was expected to produce reliable data for the incidence of frequent adverse events.[14] Although the safety data were roughly similar to those reported in the Oxford trial, with headache, fatigue, and malaise being the most common adverse events, the frequency of adverse events was higher in the Lausanne study. Only 5% (n=2) of participants had objective fever in the Oxford study, compared with 28% of participants in the Lausanne study. Overall, the ChAd3-ZEBOV was safe and well tolerated, although mild to moderate systemic adverse events were common. All vaccine recipients had humoral immune responses that peaked at day 28, and then decreased by about half 6 months after vaccination but still significantly present.

The Lausanne trial was the only one that was placebo-controlled, allowing for the most accurate assessment of safety and reactogenicity. Among all Ebola vaccine trials, this is the only one that has so far reported safety and immunogenicity results up to 6 months after injection, which provides some insight into the value of the vaccine over the course of an epidemic. When compared with results of the rVSV-vectored Ebola vaccine at 20 million or 50 million PFU, the safety profile of the ChAd3-ZEBOV at doses of 10^{10} pu is slightly better, but the humoral responses 1 month after injection are slightly lower. In view of the good safety profile of ChAd3-ZEBOV at doses of 10^{10} pu in the present trial, the authors state that the 10^{11} pu dose would seem appropriate to use when proceeding to phase 2 and 3 trials in Africa as planned, especially because the few available safety data with ChAd3-ZEBOV at 10^{11} pu show an acceptable adverse events profile and, more importantly, similar Ab responses, as those obtained with the 20 million PFU dose of the rVSV-ZEBOV. Assuming that the anti-GP antibody concentration is correlated with protection, the promising efficacy results

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reported in the preliminary report of the rVSV-vectored vaccine in the phase 3 trial in Guinea could also be obtained with the ChAd3-ZEBOV vaccine at a dose of 10^{11} pu. The persistence of Abs at month 6, although at a reduced concentration, might suggest that some protection remains. However, this theory needs to be confirmed in a thorough phase 3 trials. Detailed correlation of immunological data and protection in NHP studies might also give some insight into efficacy, if a phase 3 trial becomes impossible to do due to an insufficient number of new cases of EVD.

PRIME-BOOST STRATEGIES

ChAd3-ZEBOV/MVA-BN-Filo

Findings from studies in NHPs have shown that both immunogenicity and duration of high-level protection against challenge can be extended by administration of a dose of MVA-encoding Zaire Ebola virus GP.[11] Then, the effect of boosting in humans with a heterologous vector, MVA-BN-Filo vaccine (developed by Bavarian Nordic A/S) – which encodes Zaire Ebola virus and Sudan Ebola virus GP, Marburg virus GP, and Tai-Forest Ebola virus nucleoprotein – was assessed in Malian and US adults.[15]

The phase 1, single-blind, randomized trial of ChAd3-ZEBOV was performed in the US and the phase 1b, open-label and double-blind, dose-escalation trial in Bamako (Mali) (trial numbers NCT02231866 (US) and NCT02267109 (Malian)).

Between October 8, 2014, and February 16, 2015, participants were randomly allocated to different single doses of IM immunization with ChAd3-ZEBOV: 91 Malians received 1×10^{10} pu, 2.5×10^{10} pu, 5×10^{10} pu, or 1×10^{11} pu; 10 US participants received 1×10^{10} pu or 1×10^{11} pu. Fifty-two of the 91 Malians received a single boost-dose of 2×10^{8} PFU of MVA-BN-Filo or placebo.

The primary outcome was safety, measured with occurrence of adverse events for 7 days after vaccination. The results in both Malian and US participants document that the 1×10^{11} pu dose of ChAd3-ZEBOV is well tolerated and significantly more immunogenic than are low doses in elicitation of anti-GP antibodies. Ninety-one percent of Malian and 60% of US participants given a single dose of ChAd3-ZEBOV attained titers that are associated with protection of NHPs. A single booster dose of MVA-BN-Filo stimulated anamnestic anti-GP antibody and CD4/CD8 T-cell responses, suggesting, by extrapolation from results in NHPs, that this booster might extend the duration of high-level protection.

With optimistic extrapolation of these results, a single 1×10^{11} pu dose of ChAd3-ZEBOV, used as part of a ring vaccination strategy, might be sufficiently well tolerated and immunogenic to be effective in interrupting Ebola virus transmission to family members and other close contacts of index patients. A heterologous prime and boost regimen consisting of a ChAd3-ZEBOV prime followed 2-3 months afterwards by a boost with MVA-BN-Filo could confer long-term protection to subgroups that need extended protection (e.g. health-care workers and populations that are likely to be repeatedly exposed).

Ad26-ZEBOV/MVA-BN-Filo

NIAID and other funding partners supported the development, preclinical and clinical testing of an investigational vaccine regimen designed to specifically protect against the Ebola virus strains responsible for the recent outbreak in West Africa. The vaccine candidate combines the Ad26-ZEBOV vector (based on the AdVac platform developed by Crucell Holland B.V., one of the Janssen Pharmaceutical Companies of Johnson & Johnson) with the MVA-BN-Filo. This product commenced a phase 1 clinical trial in Oxford during January 2015. Preliminary data from the first-in-human study, presented by Janssen in May 2015, indicated that the prime-boost vaccine regimen is immunogenic, regardless of the order of vaccine administration, and provoked only temporary adverse reactions normally expected from vaccination.[16]

Additional phase 1 trials are underway in Africa. In July 2015, Crucell initiated a phase 2 clinical trial of the investigational vaccine in the UK and France. The study is evaluating the safety, tolerability and immunogenicity of the heterologous prime-boost regimen. In total, the studies enrolled 612 healthy adult volunteers, all receiving the Ad26-ZEBOV prime or placebo on day 1 and then the MVA-BN-Filo boost or placebo on days 29, 57 or 85.

In October 2015, Crucell launched a second phase 2 study in 1,200 volunteers in Sierra Leone. The first stage of the study called "EBOVAC-Salone" includes approximately 40 adults aged 18 years or older. In stage 2, approximately 400 individuals across different age groups will be vaccinated, including children and adolescents.[17]

CONCLUSION

The recent Ebola epidemic has galvanized the development of filovirus vaccines and presented a unique set of challenges that required extraordinary collaboration, flexibility, and innovation among a number of entities with a broad range of expertise to secure global health. These entities included government agencies, especially health officials from the affected West African countries, non-governmental organizations, academic research groups, pharmaceutical companies, and the WHO. This lead to an unprecedented speed of Ebola vaccine testing since late 2014 with multiple candidates in advanced stages of clinical development.

Current efforts to develop a vaccine are focused on the viral GP encoded by the virus. The most advanced first-generation Ebola vaccines candidates tested so far are the live replicating rVSV-ZEBOV and the replication-defective ChAd3-ZE-

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BOV based on the GP from the Zaire strain of Ebola virus. Trials being undertaken in Africa, Europe and US have already shown that these vaccines are safe and well-tolerated and, although a human correlate of protection remains unknown, a single dose of rVSV-ZEBOV or ChAd3-ZEBOV is able to generate putatively protective Ab titers. ChAd3 vaccine on its own especially at 10^{11} should be effective as it produces Ab titers at least as high as VSV and greater cellular immunity (which is relevant at least in NHPs).

These immune responses are significantly enhanced in prime-boost regimes using MVA-based virus vectors as a boosting vaccination, although the optimal interval between the priming and boosting vaccination has not yet been determined.

Although the promising efficacy of rVSV vaccine is encouraging, challenges remain in improving vaccines to provide durable efficacy and identifying optimal ways for vaccine deployment. Moreover, as a general rule, live-replicating viruses such as VSV-vectored vaccines are contraindicated in people with an immunodeficiency (e.g. HIV^{+}) and children, since the vaccine strain could be pathogenic, particularly when non-replicating viral vectors such as adenoviruses have shown good safety in those two groups. Clinical assessment should continue to allow a full comparison of ChAd3 alone, ChAd3/MVA, Ad26/MVA and rVSV for safety, with particular emphasis on the rate of post-vaccination fevers and arthritis, and for immunogenicity, preferably in African populations where the vaccine will be needed in future.

Some 300,000 doses of rVSV-ZEBOV vaccine against EVD will be available from May 2016 for use in emergency situations and clinical trials, under a deal signed by the drug company Merck and by Gavi, a vaccine provider to the poorest countries in the world.

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Commentary

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C-C Chemokine Receptor Seven (CCR7): Coming of Age In Vaccines

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In casual conversation with non-medical individuals, it is common for them to ask: why do we not have a cure for cancer or vaccinations for all diseases? It seems somewhat logical to assume that after so many years of research that cures should be readily available, diseases in general should simply require a pill or jab and that somehow, if scientists are not deliberately hiding these cures, then they must be asleep at the wheel. A typical response to such questions focuses on the complexity of the different cancers/diseases and that there will be no "one cure fits all". When it comes to vaccinations, there is absolutely no doubt that many vaccines are extremely effective and a multitude of publications can attest to this and cite how many lives have been saved because of our vaccination programs; indeed, vaccinations typically pop up on a list of reasons why humans today are living substantially longer than at any previous time in history.[1-3] Nevertheless, there is always an overriding and to some extent embarrassing realization that despite the relative success of vaccines we still do not, for the most part, know how to make consistently effective vaccines and that often it boils down to a trial and error procedure to establish the best vaccine for a given target.

While vaccine design incorporates a multitude of factors, one way to boost the effectiveness of a given vaccine is to enhance the secondary immune response to provide a more robust production of long-lasting antibodies, primarily IgG's. Although a discussion of the secondary immune response could take up a substantial review, in this short commentary as an example of one way to potentially boost antibody production, we will focus on a single chemokine receptor, C-C Chemokine Receptor Seven (CCR7). CCR7 is expressed on a number of cells, in particular cells of the immune system including naïve and central memory T-cells, activated B-cells, monocytes, neutrophils and mature dendritic cells.[4-6] CCR7 has two chemokine ligands, CCL19 and CCL21 that are primarily expressed in secondary lymphoid organs and plays a vital role in the chemotactic migration of CCR7 expressing immune cells to the secondary lymphoid tissues.[7] It was reported in $CCR7^{-/-}$ BALB/c mice that the migration of B-cells, T-cells and mature dendritic cells was severely compromised and that the architecture of the secondary lymphoid organs was significantly altered when compared to the wild-type strain.[5] Furthermore, 10 days after exposure to the T-dependent antigen, DNP-KLH, wild-type mice produced a robust primary immune response, in contrast $CCR7^{-/-}$ mice had a significantly compromised humoral response; however, after a further 10 days production of IgG1, IgG2a, IgG2b and IgG3 was similar for both the wild-type and $CCR7^{-/-}$ mice.[5] Fourteen days after a booster immunization the $CCR7^{-/-}$ mice showed an elevated IgG2a and IgG2b titer compared to wild-type mice;[5] (our own unpublished studies using $CCR7^{-/-}$ C57BL/6 mice). These studies demonstrated that CCR7 deficient mice have a pronounced delay, but eventual enhancement in IgG isotype switching.

A subsequent study used the paucity of lymph node T-cells (plt) mice, in which a spontaneous mutation resulted in a loss of expression of CCL19 and CCL21 in secondary lymphoid organs and a defect in homing of naive T-cells to these tissues.[8] The plt mice also demonstrated a striking attenuation in the migration of activated dendritic cells to the T-cell zones of spleen and lymph nodes.[7,8] In general abnormalities in leukocyte migration were more severe in $CCR7^{-/-}$ mice compared to the plt mice suggesting that there are functional immune differences between the plt and $CCR7^{-/-}$ mice. After immunization of plt mice, T-cells and dendritic cells mislocalized in the lymph nodes and spleen compared to wild-type

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mice; however, similar to the results for the CCR7$^{-/-}$ mice plt mice mounted an enhanced, but delayed T-cell related humoral response.[8] Interestingly, CCR7$^{-/-}$ mice have an increased polarization of CD4^{+} T-cells towards a TH2 phenotype and B-cell activation exemplified by an upregulation of MHC class II surface molecules, which points at a potential humoral response target.[9]

A primary question is whether T-cell dependent activation is required to elicit a delayed and enhanced antibody production in mice with a compromised CCR7 pathway and evidence suggests that this is the case. Thymus-independent type 2 (TI-2), are repetitive antigens, which elicit antibody production by B-cells without T-cell involvement.[10,11] Immunization of CCR7$^{-/-}$ mice with TI-2 antigens resulted in an increased number of germinal centers in the spleen that persisted for longer periods when compared to wild-type mice, although there was no increase in germinal centers of the lymph nodes in TI-2 treated CCR7$^{-/-}$ mice.[12] The persistence of germinal centers in the spleens of CCR7$^{-/-}$ mice was not associated with elevated secondary antibody responses, isotype switching, affinity maturation or memory B-cell generation.[12]

Considering that CCR7 is often discussed in the context of homing T-cells, B-cells and mature dendritic cells to the secondary lymphoid organs, it is empirically surprising that a robust T-cell response has been observed upon loss of the functional chemokine receptor or loss of both activating ligands, albeit that there are not completely overlapping immune manifestations. These responses occur in the absence of normal T-cell distributions and normal secondary lymphoid organ structures.[5,6,8] Along with other laboratories, we have demonstrated both shared and distinct biased signaling pathways in immune cells upon binding of either CCL19 or CCL21 to CCR7[13-15] recent review by Hauser and Legler.[16] It is unclear what individual effects CCL19 or CCL21 plays in modulating the secondary immune response and it will be interesting to determine what overlaying and distinct responses each chemokine contributes. Enhancing the secondary immune response in a typical boost schedule of vaccinations is an attractive target for research and this commentary touches on one chemokine in a complex wheel of factors involved in controlling the level and longevity of actions of specific antibodies. We still have a lot to learn, but the future is bright and we anticipate that this new journal will provide an avenue for novel and exciting work in the field.

CONFLICTS OF INTEREST: None.

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Research

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Herd Immunity Conferred By Hepatitis B Vaccination Increases the Protection Efficacy against Hepatitis B Virus Infection

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ABSTRACT

Most of the chronic infections with Hepatitis B Virus (HBV) are acquired in perinatal period or in early life in China. We conducted HBV serosurveys in the same community among the age 5-6 year old children who were born in 1979-1980, in 1985-1986, and in 2002-2003, respectively. The seropositive rate of HBV surface antigen (HBsAg) was 11.43% in the unvaccinated population. It decreased to 2.08% among the vaccinated children born in the same year. With increased vaccination coverage the HBsAg seropositive rate decreased to 0.24% among the age 6-7 year old children born in 2002-2003, with 98.21% protection efficacy. Herd immunity conferred by HBV vaccination increases the protection efficacy against HBV infection.

KEYWORDS: Hepatitis B virus; Hepatitis B virus infection; Primary liver cancer; Vaccine protection.

INTRODUCTION

Hepatitis B Virus (HBV) infection is one of the leading cause of illness and death in China.[1] Primary Liver Cancer (PLC) and liver cirrhosis are the long-term major adverse outcomes of chronic HBV infection.[2] In the Chinese population, most of the chronic infections are acquired in perinatal period or in early life. In 1992 before the national HBV vaccination program, seropositive rate of HBV surface antigen (HBsAg), that reflect the status of chronic HBV infection, in the 1-4 age group was 9.67%, as high as in the general population (9.75%).[3] In response to the recommendation from a WHO scientific group about the prevention of chronic HBV infection and PLC,[4] China implemented the universal immunization to newborns by integrating the HBV vaccination into the Expended Program of Immunization (EPI), beginning in January, 1992 with 3 doses of vaccines paid by the family.[5] From January 1, 2002 the HBV vaccination was integrated into the national EPI program with the vaccine provided entirely by the government. Here we reported the effect of herd immunity after HBV vaccination on HBV infection among children.

METHODS

Study Population

The children aged 5-6 years who were born in different years and resided in Qidong county, Jiangsu Province of China were recruited for the study. Serum samples were collected in the following years, respectively. In 1985 just before the Hepatitis B vaccine was introduced into China, a total of 433 blood samples from the children born in 1979-1980 were collected.

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In 1991 before the Expended Program of Immunization (EPI), a total of 3002 children, who were born in 1985-1986 and had the record of not receiving the vaccine, donated the blood samples. In addition, 674 children, who were born in 1985-1986 and had the record of receiving 3 dose of the plasma-derived hepatitis B vaccination after birth, donated the blood samples. In 2008 a total of 823 children born in 2002-2003 when all the neonates received obligatory hepatitis B vaccination after birth donated blood samples.

Serological analysis for HBV infection

Upon receiving a written consent from the children's parents, 2 ml peripheral blood was collected from each of children. All HBV serological markers were determined within 12 hours after blood sampling. Individuals with serum HBsAg-positivity were retested in six months. All the serum HBsAg was detected using the reagents from Abbott Laboratories, North Chicago, IL, USA.

Statistical analysis

Chi-square test and Fisher's exact test were conducted to compare HBsAg seropositive rates. Vaccine protection efficacy is calculated based on the HBsAg seropositivity determined among the children born in 1979-80 (as the reference group) by the form: HBsAg(+) rate in reference group -HBsAg(+) rate in intervention group)/ HBsAg(+) rate in reference group.

RESULTS

HBsAg Seroprevalene among the Children in Different Period

We conducted HBV serosurveys among the children aged 5-6 years in different period. The using of disposable medical materials and having the baby delivered in hospital began from 1980s in Qidong, one of the rural areas with HBV high prevalence. The seropositive rate of HBsAg was 13.39% among the children born in 1979-1980 and was 11.86% among those born in 1985-1986 when no vaccination was given after birth (Table 1). Chi-square test showed no difference among the children born in these two different years (p=0.3587).

Protection Efficacy of HBV Vaccination on the Children in Different Period

The Expended Program of Immunization (EPI) began in January, 1992. Some of the children born in 1985-1986 received the hepatitis B vaccination after birth. It was found that the HBsAg sero-positive rate (2.08%) decreased dramatically in the vaccinated children compared to those children without vaccination (11.86%) who were born in the same year. The protection efficacy of HBV vaccination was 84.47%. With increased vaccination coverage among the population the HBsAg seropositive rate (0.24%) further decreased, with 98.21% protection efficacy.

DISCUSSION

With the using of disposable medical materials and having the baby delivered in hospital the HBsAg-seroprevalence decreased in the HBV highly prevalence area. However, the most important strategy to protect the children from HBV infection was proven to be the neonatal HBV vaccination. Vertical transmission is a major route for HBV infection in Asian countries and endemic areas, which accounts for about 40-50% of HBsAg carriers in Taiwan and in mainland China.[3,6] However, horizontal transmission through close contact among children and family members is also a critical route for HBV infection.[6] Our results showed that HBsAg-seropositive rate was further reduced among the population when all the neonates received obligatory hepatitis B vaccination after birth. Thus herd immunity by vaccination is critical to protect population from HBV infection.

The nationwide HBV sero-survey among the mainland of China conducted in 2006 showed that the HBsAg-seroprevalence was 0.96% in the population aged 1-4 years, 2.32% aged 5-14 years, 5.4% aged 15-19 years, and more than 8.0% aged 20-59 years.[7] In 2014, the HBsAg-seroprevalence decreased to 0.32% in the 1~4 age group, to 0.94% in the 5~14 age group and 4.38% in those aged 15~29 years.[8] These data highlighted the importance of national wide neonatal HBV vaccination in reducing the HBV infection in child and adults.

Group	Year Born	Year Sampled	Age Determined	Total Num	HBsAg(+) Number	HBsAg(+) Rate (%)	*P* value	Protection Efficacy
No vaccination	1979-80	1985	5-6 years	433	58	13.39	0.3587[a]	Reference
No vaccination	1985-86	1991	5-6 years	3002	356	11.86		11.43%
vaccination	1985-86	1991	5-6 years	674	14	2.08	0.0006[b]	84.47%
vaccination	2002-03	2008	6-7 years	823	2	0.24		98.21%

[a]Chi-square test
[b]Fisher's exact test

Table 1: HBsAg seroprevalence in the children aged 5-7 years living in Qidong in different years.

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Review

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Innovations in Microbial Biodiscovery, Targeting Silent Metabolism and New Chemical Diversity

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ABSTRACT

It is quite evident that microbial (bacteria or fungi) crude extracts contain several important bioactive compounds and some have already shown their therapeutic activity. Unfortunately, most of the compounds have not properly been evaluated for the exploration of new lead molecule. Moreover, some of the mechanisms of actions of few bioactive compounds have not been identified so far. Hence, extensive research is required to find out the activity of compounds in the microbial crude extracts and to exploit their therapeutic potential to unlock silent secondary metabolites. Therefore, this review article raise the importance of activating microbial secondary metabolites noting the need for new tools to access the full microbial genome.

KEYWORDS: Microbial biodiscovery; Antibiotics; Anticancer; Silent metabolism; Lipopolysaccharide.

ABBREVIATIONS: MRSA: Methicillin-resistant staphylococcus aureus; VRE: Vancomycin-resistant enterococcus; CCR5: C-C chemokine receptor type 5; CD4: Cluster of differentiation 4; AIDS: Acquired immunodeficiency syndrome; DSB: Derivative of betulinic acid; DNA: Deoxyribonucleic acid; LPS: Lipolysaccharide; FDA: Food and Drug Administration.

INTRODUCTION TO MICROBIAL BIODISCOVERY

Historical Impact of Microbial Natural Products

Natural products continue to play a crucial role in the discovery of new drugs and drug leads.[1] Biodiscovery from microbial resources can be defined as the exploration of microbial metabolic products that provide important benefits to the fields of medicine, agriculture and biotechnology.[2] It has been reported that approximately 50% of approved drugs in the market are derived from microbial origin (Table 1).[3] Actinomycetes and fungi are very important groups of microorganisms that are known for their ability to produce secondary metabolites with therapeutic activities.[4] Actinobacteria produce over half of the bioactive compounds that are present in the antibiotic literature.[5] These compounds include well-known antibacterials such as aminoglycosides and tetracyclines, antifungals such as amphotericin B (1) anticancer agents such as adriamycin (2) immunosuppressants such as tacrolimus (3) and anthelmintics such as avermectin (4) plus numerous other valuable therapeutic drugs. Fungi also play a vital role in the microbial discovery, with the genus *Penicillium* providing several well-known drugs (e.g. penicillins). Therefore, the drug discovery process is considered as a very critical issue in the industrial field as it is a very expensive process and it requires multiple steps to produce new drug potentials to target specific disease.[6]

Despite this great success in the discovery of valuable antibiotics and other pharmaceutical active agents, there has been a dramatic decline in the discovery of new antibiotics

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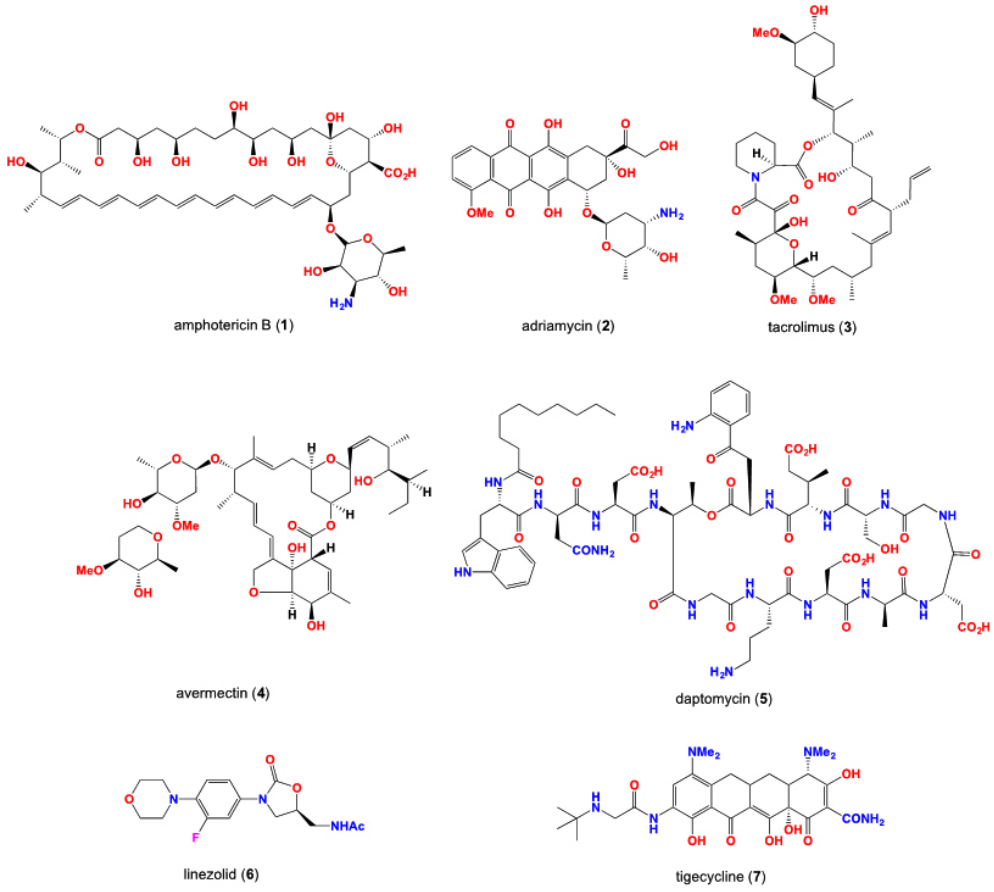
Original metabolite	Commercial products	Producing organism
Penicillins	Penicillin G, V, Ampicillin, Methicillin	*Penicillium* spp., *Aspergillus* spp.
Cephalosporins	Mefoxin, Ceclor	*Acremonuim* spp.
Thienamycin	Primaxin, Invanz	*Streptomyces cattleya*
Erythromycin	Erythrocin, Zithromax	*Saccharopolysporaerythraea*
Vancomycin	Vancocin	*Streptomyces orientalis*
Fosfomycin	Monuril	*Streptomyces fradiae*
Daptomycin	Cubicin	*Streptomyces roseosporus*

Table 1: Examples of marketed antibiotics originated from microbial origin.[3]



in the 21st century. Despite a 2007 review[7] revealing that 70% of hospital-acquired infections in the US are resistant to one or more antibiotics, with the exception of the narrow spectrum antibiotic daptomycin (5) and linezolid (6) very few new classes of clinically relevant antibiotics have been approved over the last 40 years. Tigecycline (7) is one of a new class of antibiotics derived from the tetracycline nucleus. This broad-spectrum antibiotic was approved for the treatment of complicated skin infections and is also active against methicillin-resistant staphylococcus aureus (MRSA), vancomycin-resistant *Enterococcus faecium* and beta-lactamase-producing bacteria as *Escherichia coli* and *Klebsiella pneumonia*.[8,9]

Therapeutic Fields Responsive to Natural Products

Microbial secondary metabolites are highly potent and selective for the treatment of many diseases. Many of these metabolites can be interpreted to be a signal molecule or defense mechanism against competitors or pathogens, an aid to the survival of the microorganism.

Bacterial pathogens: Natural products are still the main source of promising new antibiotics for the treatment of bacterial diseases. Platensimycin (8) was first reported in 2006 from *Streptomyces platensis* by Merck researchers, followed by the isolation of platencin (9).[11-13] In 2008, another platensimycin analogues B_1-B_3 (10-12)[14] and platensimycin B_4 (13).[15] This class of antibiotics demonstrated a novel mode of action against gram-positive bacteria through inhibition of cellular lipid biosynthesis. Because of this unique role, it shows no cross resistance to MRSA, vancomycin-resistant enterococcus (VRE) or other antibiotic resistant microbes.

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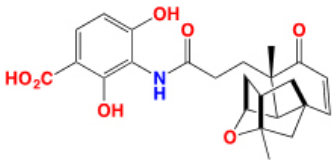
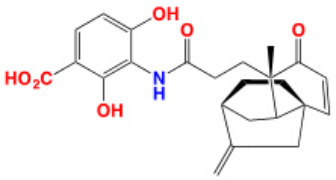
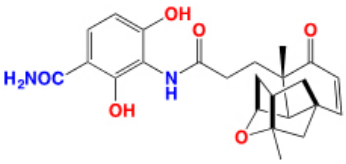


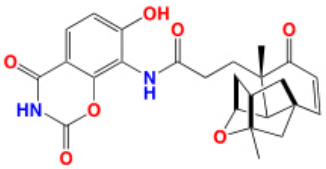
platensimycin (**8**)



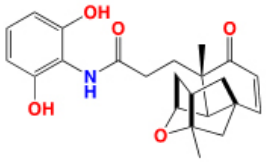
platencin (**9**)



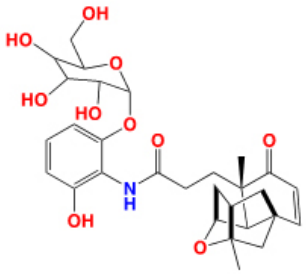
platensimycin B_1 (**10**)



platensimycin B_2 (**11**)



platensimycin B_3 (**12**)



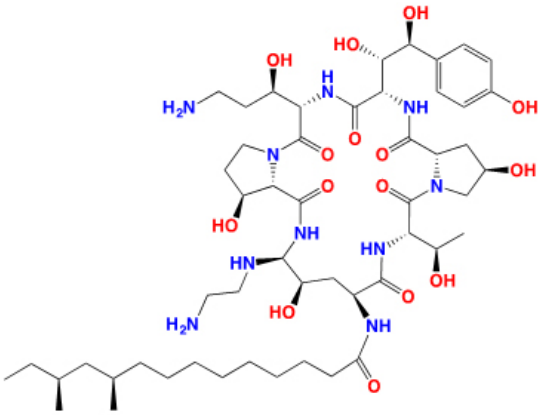
platensimycin B_4 (**13**)

Fungal pathogens: The need for the discovery of new antifungal agents continues, as we seek to treat the opportunistic infections in immune-compromised patients and the emerging resistance to existing antifungal agents. Jacob et al,[16] recently discussed the whole cell screening techniques targeting natural products and their mode of action including the use of genetically modified fungal strains in which the antifungal drug is targeting the cell wall and cell membrane. Caspofungin (14) is a promising new antifungal agents derived from pneumocandin, a natural product produced by *Glarealozoyensis*.[17]

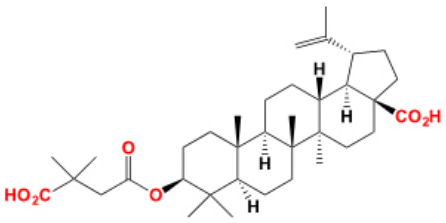
Viral pathogens: New anti-HIV (human immunodeficiency virus) drugs are targeting the point of entrance of the virus into the cell such as interferon signaling and cell surface receptors as C-C chemokine receptor type 5 (CCR5)-human cytokine receptor and cluster of differentiation 4 (CD4).[18] Many antiviral lectins are from algal origin and they are mainly small proteins that bind to carbohydrates found on the viral envelopes and prevent the transmission of HIV.[19] In addition, viral polymerases are the key for viral replication and are considered as promising targets for anti-viral drugs, with inhibitors for viral polymerase and proteases in clinical use for the treatment of acquired immunodeficiency syndrome (AIDS) and chronic hepatitis. All drugs that are in the market for the treatment of HIV are synthetic in origin, although the activities of many natural products have been explored in the recent years.[20] Yu et al,[21] discovered a plant-derived modified betulinic acid derivative, DSB (15) as a first in class HIV maturation inhibitor. This natural product is currently in Phase II clinical trial.[21]

Resistance to Microbial Natural Products

Antibiotics and the resistance in nature: The discovery of antibiotics more than 70 years ago initiated an era of drug innovation in human and animal health. These discoveries were interrupted by the emergence of antibiotic resistance, largely due to the widespread of overuse of antibiotics in both medicine and agriculture.[22] Not withstanding the outstanding list of antibiot-



caspofungin (**14**)



DSB (**15**)

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ics that were discovered from microbial sources, especially soil microbes, resistance to these antibiotics evolved in the environment long before they were developed as commercial antibiotics.[23] The resistance we see in the clinic today is to a great extent similar to the environmental one. Although the resistance in both contexts are similar, clinical resistance tends to increase substantially with time. Therefore there should be a certain mechanism that keeps control of resistance in the environment.[24-26]

Anticancer resistance: Cancer cells become resistant to anticancer drugs by several mechanisms. One way is to pump drugs out of cells by increasing the activity of efflux pumps, such as ATP-dependent transporters. Alternatively, resistance can occur as a result of reduced drug influx–a mechanism reported for agents that interact with intracellular carriers or enter the cell by means of endocytosis. In cases where drug accumulation is unchanged, activation of detoxifying proteins, such as cytochrome P450 mixed-function oxidases, can promote drug resistance. Cells can also activate mechanisms that repair drug-induced deoxyribonucleic acid (DNA) damage. Finally, disruptions in apoptotic signalling pathways (e.g. p53 or ceramide) allow cells to become resistant to drug-induced cell death.[27]

In order to overcome these problems, we need to better understand the microbial genomics. In the last 10 years, advances in genomics have revealed new knowledge of a silent microbial secondary metabolism, offering a glimpse of a new source of potentially valuable biomedical agents and tools. To access this genetic resource requires molecular tools capable of activating latent secondary metabolism gene clusters, to build knowledge of microbial systems biology, and facilitate access to new microbial natural products.[28-30]

Silent Resources

After 100 years of discovering microbes, there is great evidence that microbes are capable of producing different classes of bioactive secondary metabolites.[31] However, strong evidence revealed that we are only scratching the surface of microbial genome. Microbes share and accumulate multiple secondary metabolite gene clusters, which are capable of producing different bioactive metabolites, with a wide range of biological activities.[32] Two challenges limit our ability to access the microbial genome; the first one is that both the bacteria and fungi harbour massive number of genes. These genes usually remain dormant and do not produce any metabolites under normal laboratory conditions until they become activated. It is very probable that these genes are activated by chemical stimuli produced by other competing microbes. Such chemical stimuli represent valuable molecular tools that could be used to unlock the silent secondary metabolism, improving prospective for next generation antibiotics.[33]

Detecting of silent secondary gene cluster: A 2001 study by Omura et al, on the genome of *Streptomyces avermitilis*, the microbial source of anthelmintic drug avermectins, revealed an 8.7 Mbp linear chromosome with 25 recognizable secondary metabolite gene clusters accounting for 6.4% of the genome. This study concluded that there are many uncharacterized genes involved in the secondary metabolism. Twenty-five secondary metabolite gene clusters were found in the genome of *S. avermitilis.* Four of them are responsible for the production of melanin pigment on solid medium, two are derived from tyrosine and one is an aromatic polyketide. Another melanin is an ochronotic pigment, which is derived from homogentiginic acid and produced, in both solid and liquid media.[34] *S. avermitilis* has the highest number of secondary metabolite gene clusters of all bacterial genomes sequenced. The production of different metabolites can be attributed to the presence of many gene clusters, which can encode for enzymes for the activation of different secondary metabolic pathway. The report concluded that there are "*many other uncharacterized genes involved n secondary metabolism*".

In 2002, Bentley et al[35] reported that the genome of *Streptomyces coelicolor* contained 20 secondary metabolite gene clusters and metabolic enzymes of known or predicated secondary metabolites. Furthermore, the genome contained an unprecedented proportion of regulatory genes that are involved in response to external stimuli and stresses. *S. coelicolor* contains duplicated genes that operate in different phases during colonial development, which if stimulated could lead to new pharmaceutical compounds. The report concluded by noting "*The abundance of previously uncharacterized metabolic enzymes, particularly those likely to be involved in the production of natural products, is a resource of enormous potential values*". In 2005, McAlpine et al[36] reported the genome analysis of *Streptomyces aizunensis* to detect the presence of hitherto unexpressed natural product in which different media where used to express microbial secondary metabolites. Udwary et al,[37] analysed the genome of *Salinospora tropica* to show a large percentage of its genome is dedicated to natural product assembly. In addition, this study identified secondary metabolic biosynthetic gene clusters from the complete genome sequence of *S. tropica* revealing an unrealised secondary metabolism potential and the importance of the understanding and the control for the secondary metabolite pathway.

Therefore, modern genomics has redefined our understanding of microbial secondary metabolism. The computational analysis of microbial metabolism provides the sequencing of thousands of microbial genomes. This knowledge will provide a new pathway to access the microbial molecular diversity and also will help to develop more tools and methodologies to take advantage of this potential. In addition, the previous articles noted a growing appreciation of a global microbial genome that encompasses molecular discovery value than previously appreciated. On the other hand, while learning that microbes possess a secondary metabolism, the challenge is how to activate and benefit from that the activation of silent secondary gene clusters and produce new pharmaceutical compounds.

Activating of Silent Genes

Effect of culturing conditions on secondary metabolite production: The choice of the cultivation parameters is crucial for the

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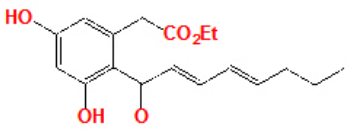
production of secondary metabolites by microorganisms. It is well known fact that a change in culture conditions (temperature, pH, oxygen etc.) can affect secondary metabolites production. Any small change in the culture medium may impact not only the level of production of certain compounds, but also the diversity.[38] Paranagama et al[39] studied the effect of tap water and distilled water on the metabolite profiling of 2 plant associated fungus, *Para phaeosphaeria quadrisep tata* by the changing the water used in fermentation from tap to distilled resulted in the production of 6 new compounds, cytosporones F-I (16-19), 5′-hydroxymonocillin (20) and quadriseptin A (21).

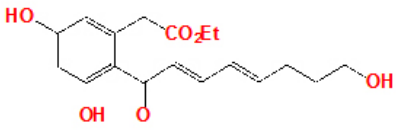
The second fungus, *Chaetomium chiversii*, produce-dradicicol (22) on solid phase media, but shifted to chaetochromin (23) in liquid phase.[39]

In addition to various culture conditions, Ayer et al have studied the impression of the diverse stress conditions on secondary metabolite production. They were able to isolate jadomycin B (24), a glycosylated nezoxazolophenanthridine antibiotic from *Streptomyces venezuelae*. The aglyconejadomycin was produced in a galactose-isoleucine medium at 37 °C.[40] Increasing the temperature to 42 °C increased the level of production of jadomycin B (24). Other parameters were found to increase the production of jadomycin B such as addition of ethanol as well as bacteriophage infection.[41] Also, Overy et al[42] cultivated different strains of necrotrophic *Penicillium* strains in different macerated host tissue media. This led to the stimulation of production of corymbiferone (25) and corymbiferan lactones I-IV (26-29).

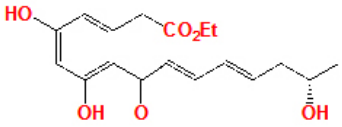
Novel metabolites in co-cultures: The functional role of natural products in microbes has long been a topic of discussion. It was found that some natural products result from the interaction of the organism with its own environment. Cueto et al cultured a marine *Pestalotia* sp. with an unidentified antibiotic resistant marine bacterium, resulting in the biosynthesis of pestalone



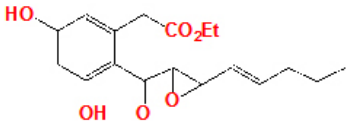
cytosporone F (**16**)



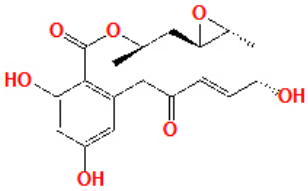
cytosporone G (**17**)



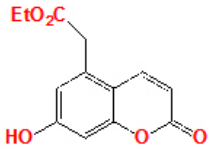
cytosporone H (**18**)



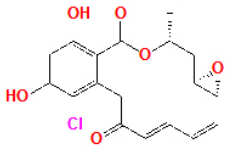
cytosporone I (**19**)



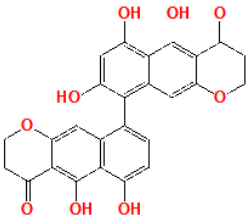
5'-hydroxymonocillin (**20**)



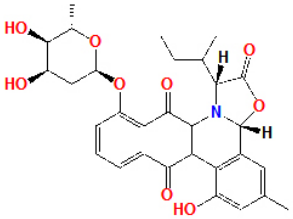
quadriseptin A (**21**)



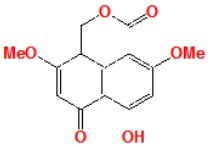
radicicol (**22**)



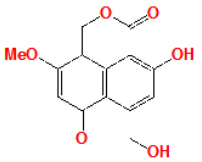
chaetochromin (**23**)



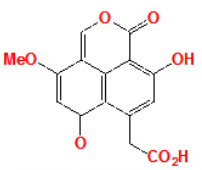
jadomycin B (**24**)



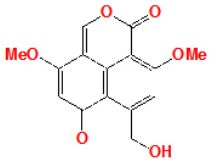
corymbiferone (**25**)



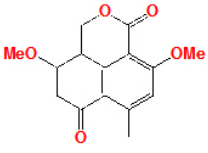
corymbiferan lactone I (**26**)



corymbiferan lactone II (**27**)



corymbiferan lactone III (**28**)



corymbiferan lactone IV (**29**)

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(30), a new benzophenone. This compound could not be detected when each strain was cultivated independently. Pestalone was found to be active against MRSA and vancomycin-resistant *Enterococcus faecium*.[43] The marine derived fungus *Emericella* sp. was challenged with the actinomycete *Salinispora arenicola*. This co-culture activated the production of two new cyclic depsipeptides, emericellamide A (31) and emericellamide B (32).[44] Latifiet al. reported another interesting example of the synergistic function of co-culturing in 2006. A co-culture between *Pseudomonas aeruginosa* and *Enterobacter* sp. stimulated *P. aeruginosa* to produce the blue pigment pyocyanin, (33).[45] To establish whether any other microorganism can induce the production of pyocyanin, *P. aeruginosa* was cultivated with different microorganisms. These results showed that *P. aeruginosalost* its ability to produce pyocyanin when co-cultured with other microbes.[46] These related examples highlight the role that co-culture can play in the regulation of secondary metabolite production.

Although these strategies have been studied and can lead to promising results, it is nevertheless a time consuming and unpredictable process that while it can express silent metabolites, is not up to the challenge of activating the broad array of silent metabolites hidden within the global microbial genome. In order to access the microbial genome, new approaches informed by the microbial evolution need to be acquired.[47]

Autoregulators: Autoregulators are compounds that act as triggers to improve cellular development and activate silent secondary metabolite genes, leading to the production of secondary metabolites. Knowing that a microbe is genetically far more capable than previously suspected, reveals a potential, but activating that potential is another challenge entirely. Even without detailed knowledge of the microbial genome, early researchers enjoyed some success in stimulating microbial secondary metabolites. For example, it had been recognized that morphological differentiation during the growth of *Streptomyces* sp. was closely associated with the secondary metabolites production. More specifically, the transition from primary to secondary metabolism occurred simultaneously with the formation of the aerial hyphae. In exploring these phenomena, a selection of low molecular weight metabolites were found to act as regulators of secondary metabolism.

γ--Butyrolactones of actinomycetes: The potential value of autoregulators as tools to regulate antibiotic production was quickly recognized, such that γ--butyrolactones came under intense investigation. An early example, of such a regulator (called an autoregulator) discovered in 1979 was A-factor (34), a γ--butyrolactone produced by Streptomyces griseus. A-factor induced both aerial mycelium formation in *S. griseus* and production of the antibiotic streptomycin, with A-factor appearing prior to streptomycin production and disappearing before streptomycin reached its maximum level.[48]

In addition to its effect on streptomycin (35) production,[49] other achievements of A-factor included inducing the production of pristinamycins in a mutant strain of *S. pristinaespiralis*, in which pristinamycins biosynthesis was otherwise blocked, and initiating the production in *S. griseus* (under phosphate depletion) of the parasiticidal metabolites grixazones A and B (36 and 37). Other closely related naturally occurring microbial **γ**-butyrolactone autoregulators included the virginiaebutenolides (38) (i.e. VB-A), which activated the production of virginoamycin that consists of 75% virginamycin M_1 (39) and 25% virginamycin S_1 (40), IM-2 (41), which activated the production of showdomycin (42) and minimycin (43), and SCB1 (44), which activated the production of actinorhodine (45) and undecylprodigiosin (46). IM-2 is noteworthy in that it both activates and suppress secondary metabolism. For instance, biosynthesis of the antituberculosis antibiotic D-cycloserine by *S. lavendulae* was completely suppressed in the presence of IM-2. Further investigations into the autoregulation of microbial secondary metabolism revealed that this capability was not limited to γ--butyrolactones.

A limited selection of *Streptomyces* metabolites have been described as promoters of morphogenesis and secondary metabolism, and include the thiazole/oxazole peptide, the polyether pamamycin-607 (47) and the siderophore desferrioxamine E (48). Pamamycin-607 (47) plays an important role in the regulation of aerial mycelium production in 67% of *Streptomyces*.[50] Desferrioxamine E (48) simulates secondary metabolites production in different strains of actinomycetes such as *Streptomyces coelicolor*.[51]

However there are some non-butyrolactone *Streptomyces* metabolites that have been discovered as promoters of secondary metabolites, including thiazole/oxazole peptide goadsporin. Goadsporin (49) is an oligopeptide consisting of 19 amino acids, which acts on the sporulation pathway and regulates secondary metabolites production for *Streptomyces*.[52]

Homoserine lactones of gram-negative bacteria: Acyl homoserine lactones (acyl-HSLs) are important intercellular signalling molecules used by many bacteria to monitor their population density in quorum-sensing control of gene expression.[53,54] These signals are synthesized by members of the LuxI family of proteins.[53] Homoserine lactones (HSLs) function by "quorum sensing" in which they reach a particular extracellular concentration due to high cell density. They act in bioluminescence, antibiotic biosynthesis, animal pathogenicity, plant pathogenicity and extracellular enzyme synthesis.[55] HSLs are similar in structure to A-factor. They can also induce exoenzymes, which are plant and animal virulence defense against *E. carotovora* and *P. aeruginosa* respectively.[56]

Genomics-Inspired Screening for Novel Natural Products

The idea of screening the extract libraries on different types of microorganisms has emerged as an important aspect to determine new metabolites.[57] Microbial metabolites (gene activators) may be capable of eliciting the expression of silent secondary metab-

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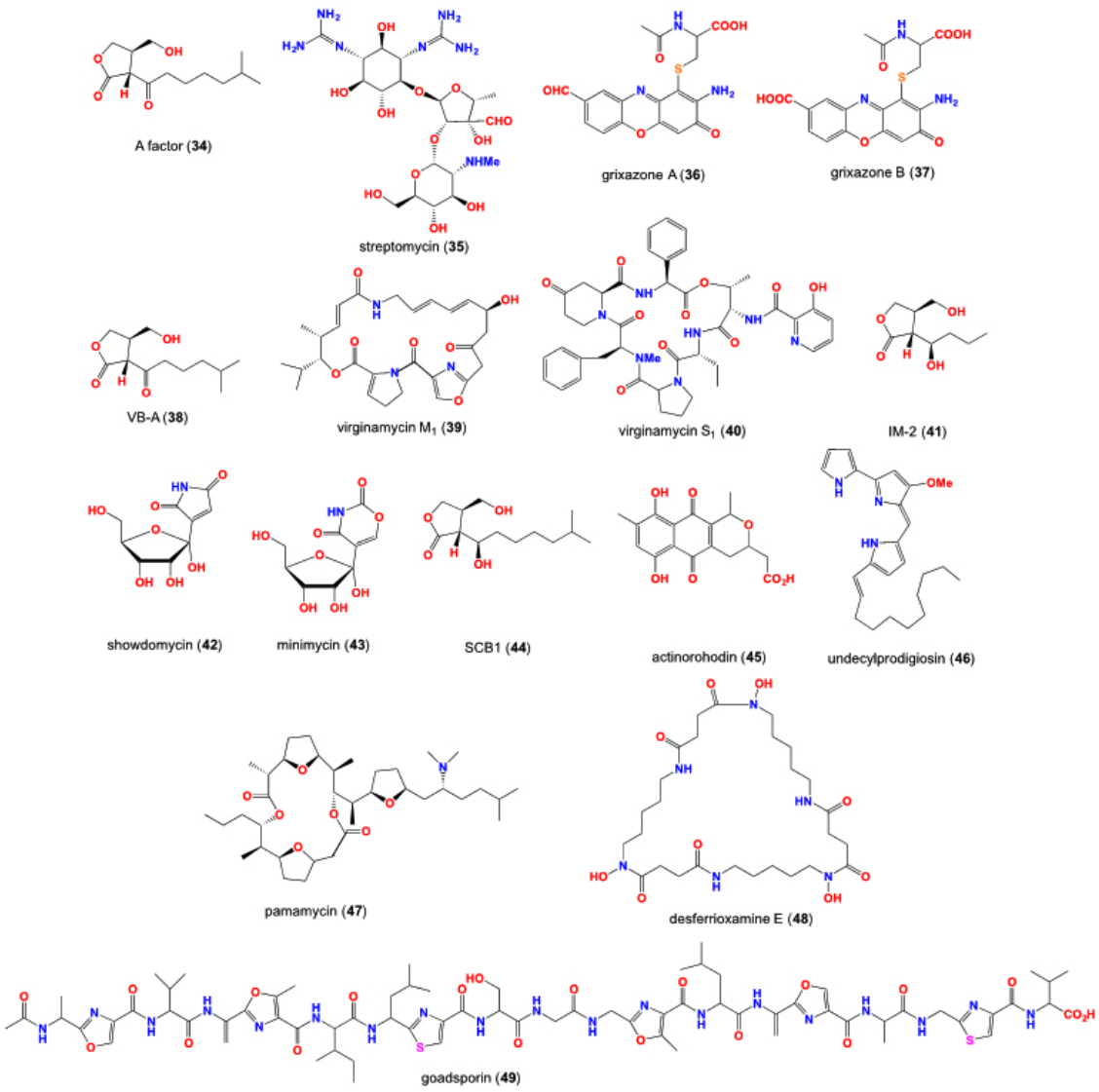

olism on other microbes. The activation of silent gene clusters will advance the discovery of next generation antibiotics.[58,59] Zazopoulos et al[60] analyzed the genome of different actinomycetes looking for the genes that were responsible for the production "enydiyne" class of antitumor agents. These loci remain inactive until certain chemical or physical signals activate them. After optimizing the growth conditions, Zazopoulos et al[60] were able to induce the expression of the gene cluster and leading to production of "enydiyne". In addition, McAlpine et al. scanned the genome of *Streptomyces aizunensis*. The genome scanning identified 11 gene clusters coding for the biosynthesis of wide range of bioactive metabolites. One of these gene clusters was responsible for the production of type I polyketide synthase generating a novel polyketide with characteristic UV absorbance at 300 nm. Varying the culture conditions helped trigger the biosynthesis of compound ECO-02301 (50).[36] Scanning the genome sequence of *Aspergillus nidulans* revealed the presence of three copies of genes that codes for proteins with high similarity to anthranylate synthases (ASs). These enzymes are responsible for the conversion of chorismate to anthranilic acid, which is important for the synthesis of tryptophan. By altering the cultivation conditions, four new prenylated quinoline alkaloids aspoquinolones I, II and III (51 -54) were produced.[61]

Epigenetic modifiers: The strategy of epigenetic modifiers (gene activators) was further studied on different fungi. Some of these studies reported that *Aspergillus* sp. contains nuclear transcriptional regulator LaeA, which controls secondary metabolite production, suggesting the existence of different regulatory mechanisms that ensure secondary metabolites production at certain developmental stages or under specific environmental conditions.[62-64] Cichewicz et al[65] treated 12 fungi with several DNA methyltransferase and histone deacetylase inhibitors. Eleven strains were found to respond, with the production of new or enhancement of known natural products. One of these strains, *Cladosporium cladosporioides*, was treated with 5-azacytidine,

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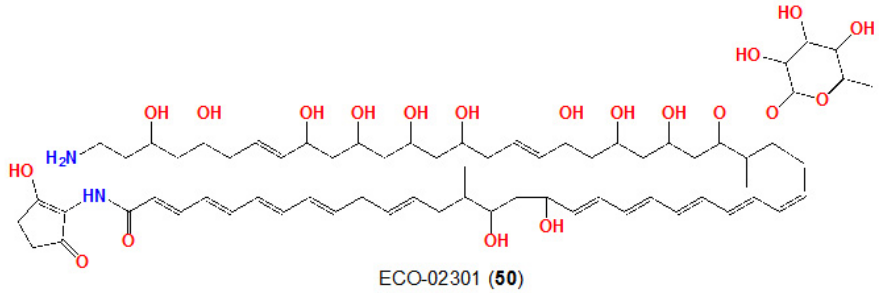
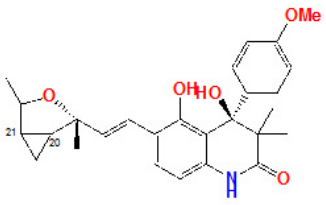
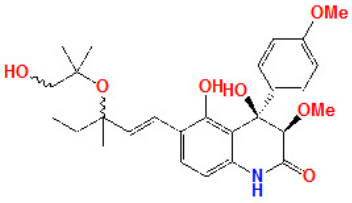


ECO-02301 (**50**)



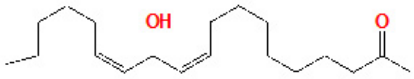
aspoquinolone I (**51**) 20 (*S*); 21 (*R*)

aspoquinolone II (**52**) 20 (*R*); 21 (*S*)

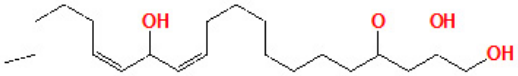


aspoquinolone III (**53/54**)

oxylipin I (**55**)



oxylipin II (**56**)



oxylipin III (**57**)

and led to the production of oxylipins I, II and III (55-57).

All these observations listed above highlight that the microbial genome offers for greater molecular potential that previously imagined, and that molecular tools are required to access this resource.

Lipopolysaccharide: The lipolysaccharide (LPS) is considered as the main component of the cell wall of all the gram-negative bacteria. The composition of the cell wall of the gram-negative bacteria is made up of an outer membrane, inner plasma membrane and a peptidoglycan layer in the periplasm. The inner cell wall is mainly considered of phospholipids while the outer surface of the outer membrane is composed of 90% LPS, plus the addition of some phospholipids and proteins. LPS consists of phospholipids in which the hydrophilic portion contains different polysaccharide made of core and outer portion.[66]

LPS is a tripartite molecule comprising a membrane-anchored lipid A moiety, a core oligosaccharide and an O-antigen polysaccharide made up of repeating units. 3-deoxy-D-manno-2-octulosonate (KDO) residues link lipid A to the core oligosaccharide, which can also be decorated with other (often non-stoichiometric) substituents, such as phosphate and phosphoethanolamine. LPS is only found in gram-negative bacteria.[67]

Bacteria with rough LPS usually have more penetrable cell membranes to hydrophobic antibiotics, since a rough LPS is more hydrophobic.[66] The hydrophobic, membrane-anchoring region of LPS is called lipid A. This part of the LPS is well conserved among bacterial species, and is considered as the most active moiety of LPS, responsible for many of the pathophysiological effects associated with infection. Lipid A is composed of phosphorylated glucosamine disaccharide decorated with 6 or even saturated acyl chains linked through amide and ester bonds. When bacterial cells are lysed by the immune system, fragments of membrane containing lipid A are released into the circulation, causing fever, diarrhoea, and even fatal endotoxic shock.[67]

Khalil et al[68] reported on a preliminary investigation of the use the gram-negative bacterial cell wall constituent lipopolysaccharide (LPS) as a natural chemical cue to stimulate and alter the expression of fungal secondary metabolism. Integrated

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HTP micro-cultivation and micro-analysis methods determined that 6 of 40 (15%) of fungi tested responded to an optimal exposure to LPS (0.6 ng/mL) by activating, enhancing, or accelerating secondary metabolite production. To explore possible mechanisms behind this effect, we employed light and fluorescent microscopy in conjunction with a nitric oxide (NO) sensitive fluorescent dye and an NO scavenger to provide evidence that LPS stimulation of fungal secondary metabolism coincided with LPS activation of NO. Several case studies demonstrated that LPS stimulation can be scaled from single microplate well (1.5 mL) to preparative (>400 mL) scale cultures. For example, LPS treatment of *Penicillium* sp. (ACM-4616) enhanced pseurotin A, and activated pseurotin A_1 and pseurotin A_2 biosynthesis, whereas LPS treatment of *Aspergillus* sp. (CMB-M81F) substantially accelerated and enhanced the biosynthesis of shornephine A and a series of biosynthetically related ardeemins, and activated production of neoasterriquinone. As an indication of broader potential, we provide evidence that cultivations of *Penicillium* sp. (CMB-TF0411), *Aspergillusniger* (ACM-4993F), *Rhizopusoryzae* (ACM-165F) and *Thanatephorus cucumeris* (ACM-194F) were responsive to LPS stimulation, the latter 2 examples being particular noteworthy as neither are known to produce secondary metabolites. Our results encourage the view that LPS stimulation can be used as a valuable tool to expand the molecular discovery potential of fungal strains that have either been exhaustively studied by, or that are unresponsive to traditional cultivation methodology.

CONCLUSION

Nature is a good source and producer of small molecules that have a great interaction with biological targets. Despite that natural products continue to provide more than half of all new drugs approved by the US Food and Drug Administration (FDA) during the last century, there is a huge shift in finding a new molecules to overcome the threat coming from multi-drug resistance bacteria and cancer resistant cells.

The decline in the pipeline for the discovery of new microbial metabolites can be attributed to the continuous rediscovery of known metabolites and we believe that the main reason for the decline in the discovery of new biologically active compounds can be that many of the microbial genes can remain dormant under normal laboratory conditions. Therefore, the microbe may require an external stimuli in order to start behave in different way and try to produce different chemistry. In fact, modern genomics have opened the tools and brighten our understanding of the microbial secondary metabolism. Medema et al[69] have demonstrated a computational analysis of the microbial metabolism through comprising approximately 300 microbial genome, which lead to the prediction of thousands of microbial genome sequencing. This great outcome provides the opportunity for the acquisition of the full microbial genome and the availability of high throughput sequencing technologies with modern analytical instrumentation allowed to determine or postulate the type of metabolites that can be produced by any microorganisms.

Therefore, we found that it is worthwhile to try to find a new paradigm for drug discovery to help to overcome the limited laboratory conditions and to establish a new technique to target the production of antibiotics. In order to perform such paradigm, we established a combinatorial chemical libraries in order to cover much chemical space to increase the probability of wakening up the silent genes and turning on new secondary metabolites.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Dendritic Cell Maturation is a Critical Step in Dendritic Cell Vaccine Preparation for Cancer Therapy

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ABSTRACT

Background: Dendritic cell (DC) vaccine is a hopeful approach for cancer treatment. In clinical trials, DC vaccines have produced clinical responses in some cancer patients. However, DC vaccines efficacy is not satisfactory in most types of cancer and more efforts must be done to improve their effectiveness in advanced cancers. Understanding the influence of tumor cells and tumor stromal cells on DCs and the antitumor activity of *ex vivo* generated DCs in the tumor microenvironment can help to augment antitumor efficiency of *ex vivo* generated DCs. In a fibrosarcoma tumor model, we explored effects of the tumor microenvironment on the antitumor efficacy of *ex vivo* generated DCs.
Methods: DCs were generated from mouse bone marrow precursor cells in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). DCs were pulsed with tumor antigens and matured in the presence of tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS), or TNF-α plus LPS. Mature or immature DCs were injected subcutaneously before tumor inoculation or were directly injected into the tumor tissue.
Results: Tumor antigen-pulsed DCs matured in the presence of TNF-α plus LPS showed appropriate functionality *in vitro*, including IL-12 secretion and induction of lymphocyte proliferation. Tumor lysate-loaded DCs matured in the presence of TNF-α did not show appropriate antitumor function *in vivo*. Injection of antigen-unpulsed mature DCs two days before tumor inoculation resulted in antitumor effects. In contrast, injection of immature DCs directly into the tumor tissue enhanced the tumor growth.
Conclusion: These results suggest that tumor cells, tumor stromal cells, or tumor derived factors can influence DCs to have tumor-promoting function. Appropriate maturation induction in *ex vivo* generated DCs and manipulating the tumor microenvironment before DC vaccination may improve antitumor activity of DC vaccines in cancer patients.

KEYWORDS: Cancer vaccine; Dendritic cell; Maturation status; Antitumor activity; Tumor microenvironment.

ABBREVIATIONS: DC: Dendritic Cell; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; IL: Interleukin; TNF-α: Tumor Necrosis Factor-Alpha; LPS: Lipopolysaccharide; APCs: Antigen Presenting Cells; MHC: Major Histocompatibility Complex; PRRs: Pathogen/Pattern Recognition Receptors; TLRs: Toll Like Receptors; RNA: Ribonucleic Acid; IU: International Unit; FBS: Fetal Bovine Serum; mAb: Monoclonal Antibody; rm: Recombinant Mouse; RBC: Red Blood Cells; PBS: Phosphate Buffered Saline; HEPES: 4-(2-Hydroxyethyl)-1-Piperazinyl-Ethane-2-Sulfonic Acid; MTT: Methylthiazolyldiphenyl-Tetrazolium Bromide; Th: Helper T; STAT4: Signal Transducer and Activator of Transcription 4; T-bet: T Box Transcription Factor; NK: Natural Killer; ELISA: Enzyme-Linked Immunosorbent Asaay; LFA-3: Lymphocyte-Function Associated Antigen-3; ICAM-1: Intracellular Adhesion Molecule-1; CD40L: CD40 Ligand; PGE: Prostaglandin E; VEGF: Vascular Endothelial Growth Factor; Tregs: Regulatory T cells; IFN-g: Interferon Gamma; IFN-α: Interferon Alpha; Poly I:C: Polyinosinic-Polycytidylic Acid; T-ALL: T-cell Acute Lymphoblastic Leukemia; IDO: Indoleamine

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2,3-Dioxygenase; TGF-β: Transforming Growth Factor-Beta; PD-L1: Programmed Death-Ligand 1; FOXO3: Forkhead Box O3; NO: Nitric Oxide.

INTRODUCTION

Dendritic cells (DCs) have a central role in body's immune system by triggering antigen-specific immune responses. DCs are the most potent antigen presenting cells (APCs) as they have a high capacity to take up antigens from the periphery, and processing and presenting them at the cell surface in the context of both major histocompatibility complex (MHC) class I and class II molecules. After antigen capture, DCs migrate to the peripheral lymphoid tissues and upregulate expression of MHC molecules as well as costimulatory molecues at the cell surface, and secrete several chemokines and cytokines to attract T cells, as well as other immune cells, and induce their differentiation towards specialized effector immune cells.[1,2] DCs are functionally divided into immature DCs and mature DCs. Immature DCs are usually found in nonlymphoid tissues and express pathogen/pattern recognition receptors (PRRs) such as toll like receptors (TLRs) to explore periphery from pathogenic agents. Immature DCs have a high capacity to capture and process antigens, but are unable to react with T cells. These cells have intracellular MHC class II molecules and express low levels of MHC class II and costimulatory molecules on the cell surface compared with mature cells. After antigen capture, they migrate to peripheral lymphoid tissues and become mature. Mature DCs express upregulated levels of MHC molecules, costimulatory molecules such as CD40, CD80 (B7-1), and CD86 (B7-2), and adhesion molecules such as lymphocyte-function associated antigen-3 (LFA-3/CD58) and intracellular adhesion molecule-1 (ICAM-1/CD54). Expression of MHC molecules and MHC-peptide complexes on DCs is 10-100 fold more than other professional APCs, including B cells and monocytes. Mature DCs can also produce several cytokines and other soluble factors. Phenotypical and functional changes occur within one day after exposure of DCs to maturation stimuli.[2,3] Therefore, mature DCs are efficient in activation of naïve T cells as well as some other immune cells. Maturation of DCs is mediated by different stimuli including microbial products such as LPS, and peptidoglycan, TLR agonists, cytokines, prostaglandins, and CD40 ligand (CD40L) expressed on T cells. TNF-α and CD40L can block differentiation of granulocytes from precursor cells and stimulate terminal maturation of DCs.[4]

Since mid-1990's, DCs pulsed with peptide antigens were used to induce antigen-specific immune responses. DCs pulsed with peptides or soluble protein induced antigen-specific cytotoxic T cells.[5,6] Because tumor specific/associated antigens are not identified in most cancers, tumor cell lysates were extensively used as a source of tumor antigens for antigen loading of DCs. Stimulation of T cells by tumor lysate-pulsed DCs led to generation of tumor-specific cytotoxic T cells.[7] Furthermore, total tumor antigen vaccines contain both MHC class I- and class II-restricted epitopes, hence, these vaccines can induce multivalent $CD4^+$ and $CD8^+$ T cell responses. In general, tumor cell lysate-pulsed DCs have produced better clinical responses than highly specific tumor vaccines. Other approaches, including tumor cell ribonucleic acid (RNA) and fusion of tumor cell to DC, have also been used for antigen loading of DCs.[4] Recently, encapsulated peptides in biodegradable nanoparticle have been successfully used to enhance DC presentation of peptides in the context of MHC class I and class II molecules.[8] Virally-engineered DCs have also been used to activate antigen-specific immune responses against murine tumors[9-11] and human cancer cell lines.[12]

In this work, we produced DCs from bone marrow precursor cells, assessed their immunophenotype and function *in vitro* as well as their antitumor activities *in vivo*, and investigate effects of maturation status of DCs and influences of the tumor environment on the antitumor efficacy of DCs.

MATERIALS AND METHODS

Tumor Cell Line and Culture Media

Fibrosarcoma cell line, Wehi-164, was cultured in RPMI-1640 medium (Gipco-Invitrogen, UK) supplemented with 2 mM L-glutamine (Sigma-Aldrich, UK), penicillin-streptomycin mixture with 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, UK), and 10% heat-inactivated fetal bovine serum (FBS) (Gipco-Invitrogen, UK). Cell culture flasks were incubated at 37 °C in atmosphere containing 5% CO_2 and 95% humidity. Cells were sub-cultured every 3 days after treatment with trypsin (Sigma-Aldrich, UK).

Antibodies and Reagents

Fluorochrome conjugated monoclonal antibodies (mAb) were used to examine DCs immunophenotype by flow cytometry. FITC-conjugated anti-mouse CD11c (N418), PE-conjugated anti-mouse I-A/I-E (M5/114.15.2), CD40 (1C10), CD80 (16-10A1), CD86 (GL-1), purified anti-mouse CD16/32 and appropriate isotype control mAbs conjugated to FITC or PE were purchased from Biolegend (CA, USA). Cytokines used for *ex vivo* generation and maturation of DCs, included recombinant mouse-granulocyte-macrophage colony stimulating factor (rm-GM-CSF), rm-interleukin-4 (rm-IL-4), and rm-TNF-α, were obtained from BD-Pharmingen (CA, USA).

Ex Vivo Generation of DCs from Bone Marrow Precursor Cells

Bone marrow cells were obtained from femur and tibia of 6 to 10 wk old BALB/c mice by flushing into RPMI-1640 medium (Gipco-Invitrogen, UK) and red blood cells (RBC) were depleted by RBC lysis buffer (Biolegend, USA). After washing, $0.7\text{-}1.5\times10^6$ cells/ml were cultured in RPMI-1640 supplemented with 2 mM L-glutamine (Sigma-Aldrich, UK), 50 µM 2-mercaptoethanol (Daejeon, Korea), penicillin-streptomycin mixture with 100 IU/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, UK), 1000 IU/ml GM-CSF (BD-Pharmingen, USA), 500 IU/ml IL-4 (BD-Pharmingen, USA), and 10% FBS (Gipco-

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Invitrogen, UK). Cell culture plates were incubated at 37 °C in atmosphere containing 5% CO_2 and 95% humidity. On days 3 and 5, fresh culture medium containing 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 IU/ml penicillin, 100 µg/ml streptomycin, 1000 IU/ml GM-CSF, 500 IU/ml IL-4, and 10% FBS were added to the plates. Morphology and immunophenotype of *ex vivo* generated DCs were analyzed by microscopically examination and immunofluorescence staining and flow cytometric analysis, respectively.

Immunofluorescence Staining and Flow Cytometric Analysis of *Ex Vivo* Generated DCs

Non-adherent cultured cells were harvested on day 6, washed two times with phosphate buffered saline (PBS), and stained with anti-CD11c, anti-I-A/I-E, anti-CD40, anti-CD80, and anti-CD86 FITC- or PE-conjugated monoclonal antibodies. After incubation at 4 °C in the dark for 30 minutes, cells were washed by PBS supplemented with 0.5% FBS and immediately assayed by a FACSCalibur flow cytometer (Becton Dickinson, USA). Acquired data were analyzed using CellQuest software (Becton Dickinson, USA). In all experiments, isotypically stained cells were used to set cursors so that the results of <1% of cells were considered positive.

Tumor Cell Lysate Preparation

Fibrosarcoma tumor cells were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FBS, as mentioned in the previous section. Cells were harvested by trypsinization, washed in PBS, and resuspended in PBS at 1×10^7 cells/ml. For preparation of tumor cell lysate, fibrosarcoma cells underwent four cycles of freezing and thawing in liquid nitrogen and 37 °C water bath, respectively. At this point, 100% of cells were seen to take up trypan blue when assessed by light microscopy. After centrifugation at 300 g for 10 minutes, the supernatants were taken and stored at -20 °C or -75 °C.

Tumor Antigen Loading of DCs

On day 6, DCs were harvested and cultured in fresh RPMI-1640 medium at a concentration of 1×10^6 cells/ml. For tumor antigen loading, tumor cell lysate was added to the DCs at a ratio of three tumor cell equivalents to one DC. Coculture continued for four hours. Some DCs did not cocultured with tumor cell lysate (unpulsed DCs).

Induction of Maturation in DCs

Maturation of *ex vivo* generated DCs was stimulated with TNF-α and/or LPS (Sigma-Aldrich, UK). After antigen loading of DCs, 5 ng/ml TNF-α and 0.5 µg/ml LPS were added to the culture medium of DCs separately or in combination. Culture incubation was continued for another 24 hours. Some DCs cultured in the absence of TNF-α/LPS (immature DCs). Maturation of DCs was assessed by flow cytometric analysis of upregulation of MHC-II molecules and costimulatory molecules CD40, CD80, and CD86 on DC cell surface.

FUNCTIONAL ANALYSIS OF DCs *IN VITRO*

Analysis of Proliferation Induction in Lymphocytes

Antigen loaded mature DCs are potent stimulator of proliferation in lymphocytes. For evaluation of this property in *ex vivo* generated DCs, DCs (2×10^4 cells/ml) were cocultured with splenocytes (2×10^5 cells/ml) obtained from naïve BALB/c mice at a responder-to-stimulator ratio of 10:1 (splenocyte:DC) in RPMI-1640 containing 15 mM HEPES (Sigma-Aldrich, UK), 2 mM L-glutamine, 100 mM nonessential amino acids (Biochrom AG, Germany), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FBS in 96 well plates. After incubation of plates for 96 hours at 37 °C in atmosphere containing 5% CO_2 and 95% humidity, MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, (Sigma-Aldrich, UK) was added to the wells and proliferation was assessed according to the manufacturer instructions.

Measurement of IL-12 Secretion

DCs can direct differentiation of naïve $CD4^+$ T cells toward type 1 helper T (Th1) cells, a major subset of $CD4^+$ T cells with antitumor activity, by secretion of large amount of interleukin-12 (IL-12).[13] IL-12 activates signal transducer and activator of transcription 4 (STAT4) signaling pathway and the transcriptional factor T-bet, which are involved in the differentiation of naïve $CD4^+$ T cells into Th1 cells. This cytokine also activates natural killer (NK) cells[14] and has potent antitumor activities.[15] Therefore, we assessed IL-12 secretion by *ex vivo* generated DCs. DCs were cocultured with splenocytes at a responder-to-stimulator ratio of 10:1 (splenocyte:DC) in RPMI-1640 containing 15 mM HEPES (Sigma-Aldrich, UK), 2 mM L-glutamine, 100 mM nonessential amino acids (Biochrom AG, Germany), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FBS. After 48 hours, culture supernatants were collected and IL-12 secretion was measured by measuring IL-12p70 in the supernatant of cultures using a commercial ELISA kite (R&D systems, Australia) according to the manufacturer instructions.

EVALUATION OF ANTITUMOR EFFECTS OF DCs *IN VIVO*

Vaccination with Mature DCs

For evaluation of antitumor effects of *ex vivo* generated DCs in *vivo*, DCs (1×10^6 cells/200 µl) pulsed with tumor antigens, or unpulsed DCs, matured with TNF-α were injected subcutaneously to the flank of female 8-10 wk old BALB/c mice (antigen pulsed DC group and unpulsed DC group, respectively). Two groups of mice were also injected with tumor cell lysate and PBS (tumor lysate group and PBS group, respectively). One other group was considered as healthy control group that did not receive any treatment (number of mice in each group was 3-5). Forty-eight hour later, fibrosarcoma tumor cells (0.7×10^6

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cells/200 µl) were inoculated subcutaneously to the same flank (previously injected site) of all mice, except for healthy control group. Tumor growth was examined every 3-4 day up to 40 days after tumor inoculation. Tumor sizes were measured by a digital caliper and tumor volumes were calculated by the formula "length×width^2×0.5".

Injection of Immature DCs Directly into the Tumor Tissue

For evaluation of the effects of the tumor microenvironment on antitumor activity of *ex vivo* generated DCs, immature DCs were injected to mouse harboring large established tumor. Fibrosarcoma tumor cells (1-1.5×10^6 cells/200 µl) were inoculated subcutaneously to right flank of mice. After 30 days, immature DCs were injected directly into the tumor tissue in three different sites. Tumor growth was measured every 3-4 day up to day 50. One group of mice received PBS and another group was considered as healthy control group (n=5).

RESULTS

Morphology and Immunophenotype of *Ex Vivo* Generated DCs

Some bone marrow cells cultured in the presence of GM-CSF and IL-4 were differentiated into DCs within two days of culture, but the numbers of DCs showing the DC morphology were increased by enhancing the culture duration time. Cells cultured in the absence of GM-CSF and IL-4 did not show any cellular projections (dendrites) and enlarged cell size. Also, the numbers of cells in these cell culture wells were diminished at the end of incubation period. In contrast, cells cultured in the presence of GM-CSF and IL-4 showed large cell sizes and enormous cellular dendrites indicative of DCs (Figure 1).

Flow cytometric analysis of cell surface expression of CD11c, MHC class II, CD40, CD80, and CD86 molecules also confirmed the generation of DCs from bone marrow precursor cells cultured in the presence of GM-CSF and IL-4. At day 7 of culture, more than 84% of cells expressed CD11c and MHC class II molecules (Figure 2). Expression of costimulatory molecules CD40, CD80, and CD86 was also upregulated at the cell surface of cells cultured in the presence of GM-CSF and IL-4. These cells did not express CD4 or CD8 on their cell surface (data did not show). At the end of culture period, 5-7×10^6 DCs were obtained from 2-2.5×10^7 cultured bone marrow cells.

In Vitro Functional Analysis of DCs

Induction of proliferation in splenocytes: Results of MTT assay showed that tumor antigen-loaded DCs matured with TNF-α or LPS plus TNF-α induced proliferation of splenocytes, but immature DCs did not. Splenocytes cultured in the absence of DCs did not proliferate. Cell counting by trypan blue exclusion test and microscopically examination also confirmed proliferation of splenocytes in the presence of mature DCs as numbers of splenocytes were enhanced 3-4 fold after four days. There was no increase in the numbers of splenocytes cultured in the absence of DCs.

IL-12 secretion: IL-12p70 was not detectable by the commercial standard ELISA kit in the culture supernatant of immature DCs cocultured with splenocytes, as well, in the supernatant of splenocytes cultured alone. IL-12p70 level was low in the supernatant of TNF-α-induced mature DCs cocultured with splenocytes. In contrast, high level of IL-12 was detected in the supernatant of LPS plus TNF-α induced-mature DCs cocultured with splenocytes (Figure 3).

***In vivo* antitumor effects of DCs:** Tumor growth rate was similar in mice received PBS, tumor cell lysates, or tumor cell lysate-pulsed DCs matured with TNF-α. But, injection of antigen-unpulsed mature DCs 48 hour prior to tumor inoculation led to delayed tumor growth in 2 of 3 mice. Injection of immature DCs directly into the tumor tissue resulted in enhanced tumor growth when compared with tumor growth rate in mice of control tumor group (Figure 4).

DISCUSSION

DCs with potent immunostimulatoy activity at the tumor sites are needed for efficient anticancer DC vaccine. Maturation and activation status of DCs robustly dictate immunostimulatory or

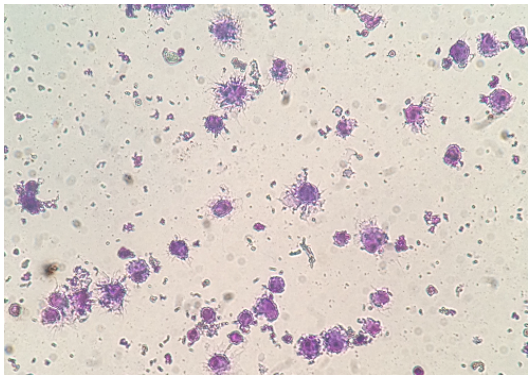

Figure 1: Bone marrow cells cultured in the presence of GM-CSF and IL-4 showed typical dendritic morphology. Cells were cytocentrifuged for Giemsa staining (×400).

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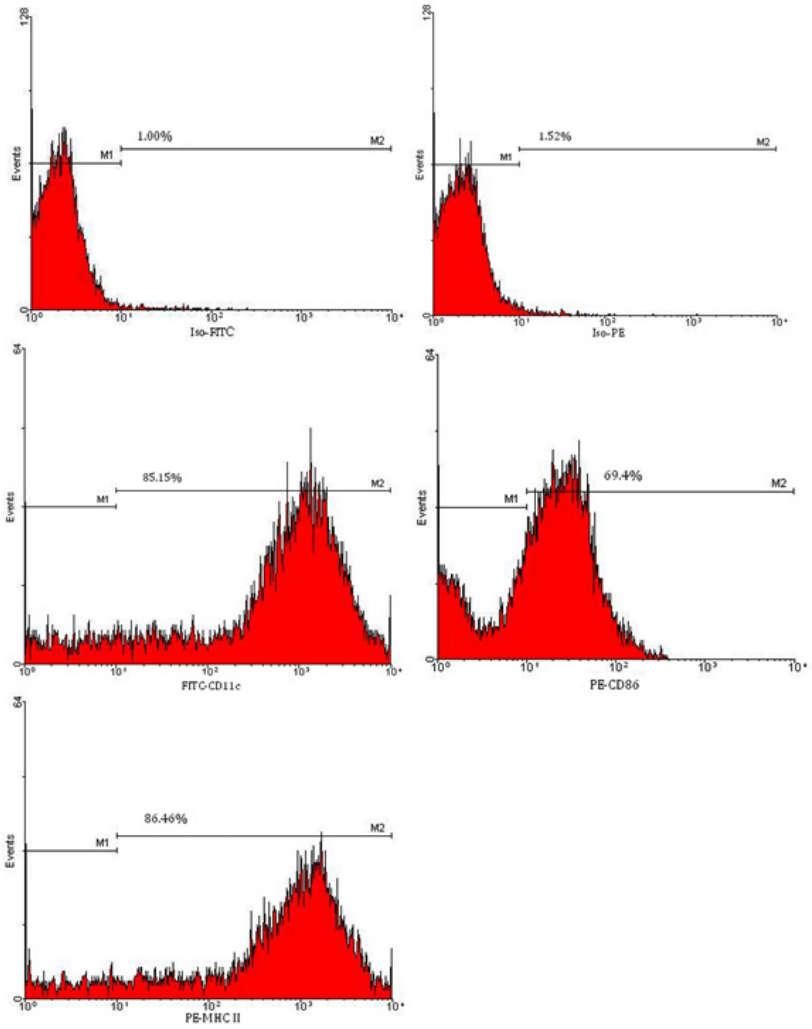

Figure 2: Flow cytometric analysis of *ex vivo* generated DCs. DCs differentiated from bone marrow precursor cells were examined for the expression of DC cell surface marker CD11c, costimulatory molecules CD86, as well as MHC class II molecules by flow cytometric analysis.

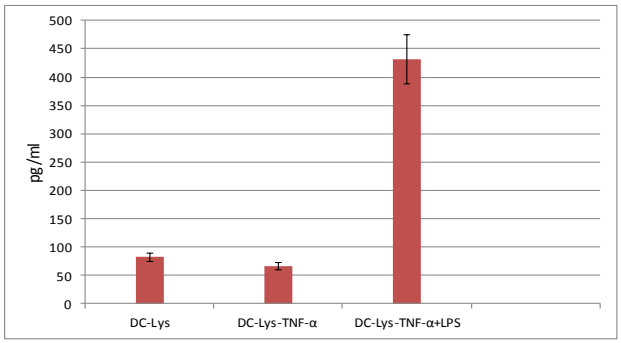

Figure 3: IL-12p70 secretion by *ex vivo* generated DCs. DC-Lys: DCs pulsed with tumor cell lysates, DC-Lys/TNF-α: DCs pulsed with tumor cell lysates and then matured in the presence of TNF-α; DC-Lys/TNF-α+LPS: DCs pulsed with tumor lysates and then matured in the presence of TNF-α and LPS.

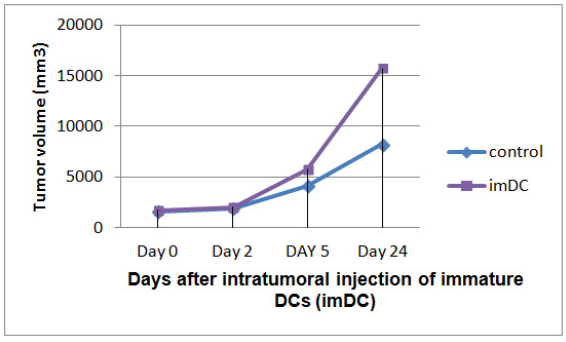

Figure 4: Intratumoral injection of immature DCs directly into established tumor tissue resulted in enhanced tumor growth rate.

immunoregulatory activities of DCs. Various maturation stimuli have been used to induce maturation in *ex vivo* generated DCs. In previous studies, a cytokine cocktail including TNF-α, interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) has been used for maturation induction in DCs.[16] However, these DCs failed to produce IL-12 and exhibited weak immunogenicity.[17] Furthermore, in later studies PGE2 has been shown to induce interleukin-10 (IL-10) and vascular endothelial growth factor (VEGF). These matured DCs were also capable of expanding regulatory T cells (Tregs),[18,19] a major immunosuppressive cell type in cancer.[20] Myeloid DCs generated in the presence of IL-1β, TNF-α, interferon-gamma (IFN-γ), inter-

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feron-alpha (IFN-α), and polyinosinic-polycytidylic acid (poly I:C) showed high migratory activity and produced high levels of IL-12p70 (the founding member of IL-12). These DCs induced up to 40-fold higher numbers of melanoma-specific cytotoxic T cells when compared to DCs matured by IL-1β, TNF-α, IL-6, and PGE2.[21] Although maturation of human DCs with a combination of IL-1β, TNF-α, IFN-γ, IFN-α, and poly I:C resulted in production of high levels of IL-12p70, however, this cocktail did not induce better T cell activation than the standard DC maturation cocktail.[22] In our 8 days culture of DCs, TNF-α induced upregulation of MHC class II and costimulatory molecules on the cell surface of DCs. Tumor cell lysate-loaded DCs matured in the presence of TNF-α plus IFN-γ produced high levels of IL-12 *in vitro* while DCs matured in the presence of TNF-α alone produced low levels of IL-12. Thus, results emphasis that appropriate maturation stimuli should be used for induction of antitumor activity in *ex vivo* generated DCs.

In various murine tumor models, intratumoral injection of DCs retrovirally transduced with IL-12 gene led to regression of day 7 established tumors (eventual rejection in 2 of 5 tumors). In contrast, intratumoral injection of non-transduced DCs did not show significant antitumor effects. IL-12-transduced DCs could migrate to tumor-draining lymph node to the same extent as non-transduced DCs. Furthermore, intratumoral injection of IL-12-transduced DCs induced more tumor-specific Th1-type responses in regional lymph nodes and spleen than non-transduced DCs.[23] In a mouse intracranial glioma model, intratumoral injection of interleukin-23 (IL-23)-transduced DCs induced effective antitumor immunity. Robust intratumoral $CD4^+$ and $CD8^+$ T cell infiltration, specific Th1-type response to the tumor in regional lymph nodes and spleen at levels greater than those of non-transduced DCs, and systemic immunity against the same tumor rechallenge were observed in animals received intratumoral injection of IL-23-transduced DCs.[24] In our study, injection of antigen-unpulsed mature DCs two days before tumor inoculation resulted in antitumor effects while injection of immature DCs directly into the tumor tissue enhanced the tumor growth.

To explain these findings, it is important to know how *ex vivo* generated DCs affect tumors, and *vice versa*. In multiple murine T-cell acute lymphoblastic leukemia (T-ALL) and primary patient samples, tumor associated DCs were identified as a key cell type in the tumor microenvironment and these DCs were necessary and sufficient to support T-ALL survival *ex vivo*.[25] In transgenic adenocarinoma of mouse prostate model, tumor-infiltrating plasmacytoid DCs expressed low levels of costimulatory ligands CD80 and CD86 and elevated levels of indoleamine 2,3-dioxygenase (IDO). DCs isolated from prostate tumor tissues were able to tolerize peripheral blood T cells and microarray-based gene expression analysis showed that the tumor associated DCs express upregulated levels of genes associated with immune suppression such as IDO, arginase, transforming growth factor-beta (TGF-β), programmed death-ligand 1 (PD-L1), and forkhead box O3 (FOXO3).[26]

In some studies, tumor infiltrating DCs have been shown to have an immature phenotype with low expression of costimulatory molecules and incapable of inducing potent antitumor immune responses.[27,28] In a murine ovarian carcinoma model, tumor progression was correlated with phenotypic changes in tumor-infiltration DCs from immunostimulatory to immunoinhibitory.[29] Melanoma-derived factors suppressed expression of costimulatory molecules CD80 and CD86 and altered production of proinflammatory cytokine/chemokine by DC lines *in vitro*.[30] Melanoma-derived factors also altered maturation and activation of differentiated tissue resident DCs.[31] In a lung metastasis model of melanoma, tumor-altered chemokine expression by lung-resident DCs correlated with increased lung infiltration by tumor-associated macrophages with M2 phenotype.[31] Freshly isolated tumor cells could derive DCs to differentiate into immunosuppressive DCs expressing high levels of IL-10, nitric oxide (NO), VEGF, and arginase I.[32]

Recently, presence of immature DCs has been found to be associated with tumor growth and angiogenesis.[33] In a mouse model of ovarian carcinoma, immature DCs contributed to ovarian cancer progression by acquiring a proangiogenic phenotype in response to VEGF secreted by engineered VEGF-A-expressing tumor cells.[34] Immature DCs expressing VEGF-receptor 2 have also been contributed to the tumor angiogensis in a murine model of endometriosis and in the peritoneal Lewis lung carcinoma tumor model.[35] DCs can help angiogenesis by producing factors that promote growth of endothelia cells.[36] In addition, DCs participated in the generation of neovessels by interacting with endothelial cells and stabilizing newly expanded vasculature at the level of lymph nodes.[37] On the other hand, DCs cultured in the presence of tumor derived factors showed some characteristics of endothelial cells suggesting transdifferentiation of DCs into epithelial-like cells.[38,39]

These findings indicate that tumor cells, tumor stromal cells, or tumor derived factors can dampen antitumor activity of DCs. On the other hand, DCs may encourage tumor growth by immunosuppressive activities or other effects such as promotion of tumor angiogenesis. Appropriate maturation induction in *ex vivo* generated DCs and manipulating the tumor microenvironment before DC vaccination may improve antitumor efficacy of DC vaccines.

CONCLUSION

For improving antitumor efficacy of DC vaccines it is important to understand how tumor and DCs influence each other. In several studies, tumor-associated DCs were immunosuppressive as they were unable to trigger tumor specific immune responses or they induced immunoregulatory cells expansion. Tumor-infiltrating DCs have also been related with tumor angiogenesis. Our results reveal that one of the main reasons why anticancer DC vaccines show limited successfulness in clinical trials may be adverse effects of the tumor microenvironment on DCs. It should be taken into account that DCs have a high plasticity,

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especially in the tumor microenvironment, and efforts must be done to improve their antitumor efficiency for example by optimal maturation of *ex vivo* generated DCs and reprogramming the immunosuppressive status of the tumor microenvironment.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ANIMAL CONSENT

In our study, we used female BALB/c mice for evaluation of antitumor effects of *ex vivo* generated DCs *in vivo*. The mice were housed in 12:12 h light-dark cycle with appropriate temperature and moisture and allowed for free access to food and water in the whole period of the study. This experiment was approved by the Institutional Animal Care and Use Committee in Tehran University of Medical Science (Tehran, Iran).

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Review

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Ebola Virus: Promising Vaccine Candidates

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ABSTRACT

Ebola Virus Disease is among the deadliest viral diseases. The field of Ebola vaccine development has progressed over the years with numerous candidates in advanced stages of clinical development. Currently, there is no licensed vaccine against Ebola virus. This review aimed to discuss the promising Ebola vaccine candidates focusing on vaccines against ebola viruses in particular against Zaire ebola virus (ZEBOV). Although DNA vaccines have been evaluated in Phase I clinical studies, it demonstrates low immunogenicity. Thus far, the most successful vaccine platforms are the recombinant viruses including, replicating vesicular stomatitis virus (rVSV) and chimpanzee adenovirus 3 (ChAd3). The successful trials attests that rVSV vaccines will be submitted for licensing in the near future.

KEY WORDS: Ebola virus; ZEBOV; rVSV; ChAd3; Vaccine; rAd5; Immunization; Clinical trials.

ABBREVIATIONS: ZEBOV: Zaire ebola virus; rVSV: replicating vesicular stomatitis viru; ChAd3: Chimpanzee adenovirus 3; EVD: Ebola Virus Disease; CIEBOV: *Cote d'Ivoire Ebolavirus*; REBOV: *Reston Ebolavirus.*

INTRODUCTION

Ebola virus disease (EVD) is among the most virulent viral infections caused by the Ebola virus, with mortality rates nearing 90%.[1,2] Ebola virus is a member of the *Filoviridae* family, and is classified into five species, *Zaire Ebolavirus* (ZEBOV), *Sudan Ebolavirus* (SEBOV), *Bundibugyo Ebolavirus* (BEBOV), *Cote d'Ivoire Ebolavirus* (CIEBOV) and *Reston Ebolavirus* (REBOV) in decreasing order of virulence.[3,4]

Ebola virus particularly, SEBOV and ZEBOV were discovered in 1976 in the Democratic Republic of Congo. Since then the virus has initiated over 20 sporadic outbreaks restricted to regions of Africa.[5,6] Consequently, in 2014, the World Health Organization (WHO) declared an urgent need for efficacy and safety testing of EVD vaccine candidates.[7]

The largest EVD epidemic to date, was reported between December 2013 and April 2016, which generated over 28,000 cases and 11,000 deaths in the African populations of Guinea, Liberia and Sierra Leone.[1,8] Up-to-date, there are no reported cases of EVD in Sri Lanka. However, due to the inevitable re-emergence of the disease, WHO has warned all countries to intensify surveillance activities to combat any possible threat of EVD. Therefore, Sri Lanka has strengthened its scrutiny activities in order to recognize, notify and manage suspected EVD patients.

The devastating effects of the 2014 EVD outbreak spurred international research into vaccine development. Although, it has been 40 years since the discovery of Ebola virus, there are no approved vaccines for the disease. Therefore, there is an urgent need for an Ebola vaccine which is cheap, effective and safe, which can be administered in a single dose.[9,10] The aim of this review is to discuss the most promising candidate vaccines which could potentially protect against the Ebola virus.

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Ebola Virus Genome

Ebola virus genome is the prime target for potential candidate vaccines. Ebola virus is a single-stranded, lipid enveloped, negative sense RNA virus of approximately 19 kb, which comprises of genes that code for seven proteins.[11] Four of the genes encode structural proteins including nucleoprotein (NP), glycoprotein (GP) and two viral matrix proteins VP24 and VP40. Whereas, VP30, VP35 and the RNA-dependent viral polymerase (L) are the non-structural proteins.[12,13] Each gene encodes functional proteins for viral replication.

Moreover, it has been recorded that vaccines for the protection against EVD predominantly target glycoprotein, with an exemption of a few directed at VP40 and nucleoprotein.[14-16]

STRATEGIES FOR EBOLA VACCINE DEVELOPMENT

The recent Ebola outbreak brought global attention to the inadequate preventive and therapeutic measures against EVD. This intern spurred intense action towards initiation and development of an Ebola vaccine. The first successful Ebola virus vaccine was a conventional inactivated vaccine developed by Lupton and colleagues in 1980.[17,18] However, due to the possible threat of incomplete inactivation *versus* using the term reversion to virulence, inactivated immunizations were not encouraged against EVD.

Over the past two decades, researchers have explored various strategies for Ebola vaccine development. Broadly, Ebola vaccine candidates can be categorised into replication incompetent and replication competent vaccines.[19,20] In the following subsections, the three most promising vaccine candidates currently undergoing clinical trials are discussed.

Replication Incompetent (Non-Replicative) Vaccines

Recombinant adenovirus based vaccines: Adenoviruses are double-stranded, non-enveloped DNA viruses, which have been isolated from mammalian species. Adenoviruses are used as recombinant vectors by deletion of the E1 region, thus making the virus replicative deficient.[21,22] Various clinical trials have demonstrated the safety of adenovirus vectors for use as a benign carrier system. In 2000, Sullivan and fellow scientists, first documented the use of recombinant Adenovirus 5 (rAd5) – based vectors which expressed EBOV antigens as a vaccine.[23]

Majority of the first generation adenovirus vectors focused on the innovation of a vaccine based on human serotype (AdHu5). However, pre-existing immunity greatly decline the efficacy of AdHu5 immunizations.[24] Several strategies have been derived to overcome pre-existing immunity, including the use of a variety of serotypes of recombinant Adenovirus such as the rare human serotypes Ad26 and Ad35. Subsequently, the second generation of adenovirus was developed from Chimpanzee Adenovirus, Ad3, Ad7 and Ad62.[25]

Initially, rAd5 vaccines were used to boost immunizations upon priming them with DNA vaccines. Although, the vaccination strategy provided 100% protection to Non-human primates (NHP) against the ZEBOV challenge, a period of over 6 months was required to complete immunization.[26,27] This study was found to be highly effective and dependent of Ebola-specific CD8+ T-cells and antibody responses. Moreover, CD8+ cells played a significant role in rAd5-GP-induced immunity against EBOV infection in NHPs.[28,29] Additionally, vaccination dose is a crucial factor in determining vaccine efficacy. Studies demonstrated that at least 1×10^{10} virus particles are necessary to attain 100% protection in NHPs against Ebola virus infections.[30,31]

In 2010, a double-blinded, placebo-controlled, dose-escalation, Phase I human study confirmed the safety and immunogenicity of rAd5 immunization encoding the envelope GP from ZEBOV and SEBOV 1976 strain. Thirty-one healthy adults were vaccinated at 2×10^{9} (n=12), or 2×10^{10} (n=11) viral particles or placebo (n=8) as an intramuscular injection. All participants demonstrated antigen specific humoral and cellular immune responses.[32]

In 2015, a placebo-controlled, double-blind, phase 1 clinical trial was performed in China, to evaluate the safety and immunogenicity of rAd5 vaccine encoding the envelope GP-ZEBOV 2014 strain. In this study, 120 healthy adults were randomly allocated to receive placebo (n=40), low-dose (n=40) or high-dose (n=40) vaccine. The results of the study demonstrated that the high-dose immunization is safe and robustly immunogenic. Single inoculation of the high-dose vaccine could mount glycoprotein-specific humoral and T-cell response against Ebola virus in fourteen days.[33]

Subsequent studies were carried out to test the rare serotype rAd vectors to circumvent Ad5 immunity. The results demonstrated that the great extent of CD8+ T-cell responses were not constantly prognostic of vaccine efficacy. In 2011, Geisbert and team identified that Macaques immunized with a single dose of rAd26 and rAd35 EBOV vaccines at the uniformly protective rAd5 vaccine dose, 10^{10} particles, produced serum anti-glycoprotein Abs along with T-cell responses of CD4+ and CD8+ comparable to rAd5. Nonetheless, this was unsuccessful in attempts to protect animals against EBOV infection.[34]

Evaluation of the cytokine secretion patterns within antigen-specific T-cells following Ebola immunization exhibited a prominent difference between rAd5 and the rare serotype rAd26 or rAd35 inoculations. It was found that the CD8+ T-cell response initiated by the protective rAd5 vaccine was significantly greater compared to the rarer serotypes, and consisted predominantly of TNF/IFN-γ double positive, cytolytic effector cells.[35]

Recently, Ad26 vaccine expressing the full-length GP of ZEBOV was administered in a prime-boost regimen with multivalent Modified Vaccinia Ankara (MVA)-Bavarian Nordic (BN) vaccine, which is currently undergoing Phase III trials.[36,37]

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Candidate vaccines based on the chimpanzee adenovirus 3 (ChAd3) platform are currently being developed by deletion and replacement of the E1 gene in the cAd3 genome with EBOV GP. Between September and December 2014, five phase 1 trials of ChAd3 commenced in North America, Europe and Africa.[38,39]

The preliminary report of the first ChAd3 trial, confirms the safety and immunogenicity of the vaccine. In this study, twenty healthy adults were enrolled in groups of 10 each, and were subjected to intramuscular immunization in doses of 2×10^{10} or 2×10^{11} particle units. All 20 participants produced Glycoprotein-specific antibodies and the 2×10^{11} particle unit dose group had a greater magnitude titer than 2×10^{10} particle unit dose group. Therefore, the immune responses and reactogenicity to cAd3-EBO vaccine were dose-dependent.[40,41]

Phase II and III trials were initiated in Liberia, Sierra Leone and Guinea. In February 2015, the trial known as Partnership for research on Ebola vaccines in Liberia (PREVAIL) was initiated.[41,42]

Furthermore, analysis of Ebola virus vaccine vectors demonstrated that robust antibody titers and high magnitude of TNF/IFN-γ double positive CD8+ T-cell quality in ChAd3 are remarkably similar to rAd5 in inoculated macaques.[43]

In 2014, researchers developed a trivalent engineered Ebola-Marburg vaccine (GreEMTri), third generational Adenovirus based vaccines, which expresses glycoprotein genes of both ZEBOV and SEBOV along with deletion of all Ad genes. Advantages of Adenovirus based vector vaccinations include, high immunogenicity, targeted immune response, multi-delivery routes and effectiveness at low doses.[44] Altogether, the available data have shown great potential for adenovirus-based vaccines to be licensed in the future.

REPLICATION COMPETENT VACCINES

Recombinant Vesicular Stomatitis Virus (rVSV)

rVSV is a member of the family *Rhabdoviridae*, and a promising vaccine platform for EBOV. In 1987, Rose et al, pioneered the use of rVSV as a vaccine vector.[45] The rVSV vector vaccine is designed to function by replacing the rVSV-G with a G from an EBOV strain by using reverse genetics.[46] This chimeric alteration attenuates the pathogenicity and the neurotropism of the rVSV delta G filovirus GP vectors for ZEBOV and MARV, while allowing the vaccine virus to replicate using the Ebola GP to attach and enter cells.[47]

In 2005, a vaccine based on rVSV was the first replicating Ebola virus vaccine shown to be protective in NHPs. In this study, a single intramuscular vaccination elicited protective immune responses in NHPs against lethal EBOV challenge. The EBOV immunization triggered humoral and cellular immune reactions in all inoculated NHPs and provided 100% protection.[48]

Furthermore, the rVSV vaccines have shown remarkable post-exposure success. In a study conducted in Canada, guinea pigs and mice were first inoculated with $2x10^4$ or 2×10^5 plaque-forming unit (PFU) of rVSV-EBOV vaccine respectively. Challenge of mice with a lethal dose of Mouse Adapted EBOV at 6.5 and 9 months after immunization proved complete protection, and at 12 months post-vaccination, 80% (12 of 15 survivors) protection. Similarly, encounter of guinea pigs with a lethal dose of guinea pig-adapted EBOV at 7, 12 and 18 months after vaccination resulted in 83% (5 of 6 survivors) at 7 months after vaccination, and 100% survival at 12 and 18 months after vaccination. The AB responses were examined using sera from each rodent. Additionally, there was a correlation between the quantity of EBOV GP-specific IgG Ab and protection. This study concluded that inoculation with rVSV-EBOV is able to confer long-term protection against EVD in guinea pigs and mice.[49,50]

Up to date, the rVSV-ZEBOV immunization has been investigated in eight human phase I trials across Europe, Africa and North America. Moreover, the Sierra Leone trial to introduce a vaccine against Ebola (STRIVE) phase III study in Sierra Leone is ongoing.[51,52]

Phase I rVSV-ZEBOV double-blind, placebo-controlled, dose-escalation trial was conducted on 52 volunteers. In this study, 12 volunteers received placebo injection and 40 participants received rVSV-ZEBOV immunization at either an intramuscular dose of 3 million PFU or 20 million PFU. The safety and immunogenicity were evaluated for 28 days post-immunization. The results demonstrated that, all participants had seroconversions by day 28, as assessed by ELISA against the GP of ZEBOV-Kikwit strain. On day 28, the group receiving 20 million PFU had a higher geometric mean titer of Ab against ZEBOV GP than the group receiving 3 million PFU. These preliminary outcomes promote the further development of the vaccine dose of 20 million PFU.[53]

A similar Phase 1/2 clinical trial in Geneva was carried out to evaluate the safety and immunogenicity of rVSV vaccine administered at different doses. In this study, 59 healthy volunteers receiving $3x10^5$ PFU low dose of rVSV-ZEBOV vaccine were compared with rVSV-ZEBOV high dose vaccines $1x10^7$ PFU (n=35) or $5x10^7$ PFU (n=16) or a placebo (n=8). Initially, viral oligoarthritis was detected in eleven of the first 51 participants (22%) subjected to receive 10^7 or 5×10^7 PFU. Thereafter, 56 volunteers received a lower dose 3×10^5 PFU (n=51) or placebo (n=5) to examine the influence of dose reduction on safety and immunogenicity. The study concluded that reduction in the dose of rVSV-ZEBOV enhanced early tolerability, nevertheless suppressed antibody responses.[54]

A Phase III efficacy trial is currently underway in Guinea to evaluate the efficacy of rVSV-ZEBOV as a preventative strategy for EVD. The trial used a ring vaccination strategy and the preliminary reports demonstrated encouraging results. In this ongoing study, 90 groups (7651 individuals) comprising

of contacts of an index case received 20 million PFU of rVSV either immediately or 21 days after documentation of the index case. In the immediate vaccination group, no cases of EVD were reported whereas 16 cases of EVD were diagnosed in the delayed vaccination group, 10 days post-vaccination. The results show a vaccine efficacy of 100%. From six days following vaccination, there were no new cases of EVD identified in vaccine recipients from the immediate or delayed groups. The data depicts that rVSV confers protection between 6-21 days after vaccination, nonetheless the longevity of the vaccine-induced protection is unknown.[55]

Advantages of using rVSV vaccines include, very limited pre-existing immunity, easily propagation in mammalian cells and capacity to initiate a strong humoral and cellular immune responses. Additionally, rVSV based vaccines have the ability to confer both systemic and mucosal immunity.[56] These clinical studies demonstrated the safety and efficacy of rVSV vaccine. Researchers predict this vaccine will be submitted for full licensing by the end of 2017.

SUMMARY

Ebola vaccine development has progressed at an exponential rate with numerous candidates in advanced stages of clinical development. Although, DNA vaccines have been evaluated in Phase I clinical studies, it demonstrates low immunogenicity and necessitates frequent vaccinations over a sustained period of time. Thereby challenging the implementation of DNA vaccine platforms as vaccination of the entire population over a long time period is logistically and financially not feasible.

Thus far, the most promising Ebola vaccine candidates are the live-replicating rVSV and the replication-defective ChAd3 based on GP of ZEBOV strain of Ebola virus. Multiple trials currently underway in Europe and Africa have demonstrated that these vaccines are safe and immunogenic. ChAd3 and Ad26 successfully passed through clinical trials and entered into a Phase III trials, whereas rVSV has been validated to be effective in a Phase III clinical studies. While the efficacy of rVSV vaccine is encouraging, challenges exist in improving vaccines to provide durable efficacy and recognizing optimal pathways for vaccine delivery. At this point, the crucial impediment for EBOV vaccines to move forward is the limited funding for vaccine development.

In summary, extensive research carried out on rVSV vaccine depicts that this persists to be the only immunization platform for prospective clinical use. Its efficacy following a single dose is a substantial benefit for providing immunizations to target populations. Moreover, this vaccine is cost-effective and is efficacious both pre- and post-exposure with a moderately short time to immunity. The next steps would be to develop a pathway to licence rVSV vaccines and potentially stockpile vaccines for future Ebola outbreaks. An effective Ebola vaccine strategy can be envisioned for use, in order to limit the spread of an outbreak and to protect individuals who are at the highest risk of infection.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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Fluorinated Lipids Conjugated to Peptide Antigens do not Induce Immune Responses Against Cervical Cancer

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ABSTRACT

Background: Despite the high safety profile of peptide-based vaccines over conventional counterparts, the inability of small peptides to produce a strong immune response represents the main obstacle for the development of these types of vaccines. Introducing a self-adjuvanting moiety such as fluorinated lipids can overcome this problem. We have recently demonstrated that fluorinated lipids can induce humoral immune responses against associated peptide antigen; however, the ability of these amphiphilic lipids to elicit a desired cellular immune response to eradicate tumor cells has not been yet investigated.
Methods: An *in vivo* assay was employed to evaluate the ability of fluorinated lipopeptides to eradicate tumor in mouse model. In this study, the double conjugation technique was used to synthesise fluorinated and non-fluorinated lipids conjugated to two cytotoxic T-lymphocyte (CTL) peptide epitopes derived from the E6 and E7 proteins of human papilloma virus (HPV).
Results: Mice implanted with TC-1 tumor cells and immunised with fluorinated lipopeptides did not mount a strong cellular immune response, thus did not eradicate the tumors. In contrast, 60% of mice immunised with the non-fluorinated lipopeptide cleared the TC-1 tumor.
Conclusion: This result indicated that fluorinated lipids lack of the ability to stimulate a strong cellular immunity despite their ability to elicit significant humoral immune responses.

KEY WORDS: Fluorinated lipid; Self-adjuvant; Cellular immunity; Peptide-based vaccine; Nanoparticle.

ABBREVIATIONS: CTL: Cytotoxic T-lymphocyte; CFA: Complete Freund's Adjuvant; CuAAC: Copper-Catalyzed Azide-Alkyne Cycloaddition.

INTRODUCTION

Vaccination is one of the most effective implements for decreasing the occurrence of infectious diseases. Peptide subunit-based vaccines have many advantages as they are more safe, stable and easy to manufacture compared to the traditional vaccines bearing live attenuated or inactivated pathogens. These modern vaccines contain only the least immunogenic section of an antigen required to elicit the desired immune response.[1] However, the use of peptides alone is not sufficient to stimulate the immune system. Therefore, an adjuvant/delivery system is the necessary component to trigger an immune response against the peptide antigen.[2]

The adaptive immune response generates a long-term pathogen-specific immune response through the generation of a broad amount of different antigen receptors and by the

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propagation of memory B- and T-cells. Cytotoxic T-lymphocytes (CTL) can kill infected cells by cytotoxic action while the B-cells produce pathogen-specific antibodies. Both of these mechanisms are assisted by T-helper cells. For production of a therapeutic and/or protective immunity, stimulation of CTLs and/or B-cells by vaccination is essential.[3,4] Recently, we tested *in vivo* the ability of peptide-based vaccines including fluorinated lipids in a conjugation with J14 B-cell peptide epitope to produce protective IgG antibodies against J14 antigen.[5] It was demonstrated that fluorinated constructs can induce effective humoral immune response once they form nanoparticles. A high antibody titer was produced by fluorinated vaccines, similar to those induced by the positive control (J14 mixed with powerful Complete Freund's Adjuvant (CFA)) and higher than those produced by non-fluorinated analogues.

Here, we synthesized two fluorinated lipo-alkynes *1* and *2* and the non-fluorinated analogue *3* as reported previously (Figure 1).[5,6] The lipid moieties were conjugated with a peptide epitopes using a copper-catalysed alkyne-azide 1,3-dipolar cycloaddition (CuAAC) reaction. The resulting conjugates were assayed *in vivo* to investigate their ability to elicit cellular immune response in mice.

MATERIALS AND METHODS

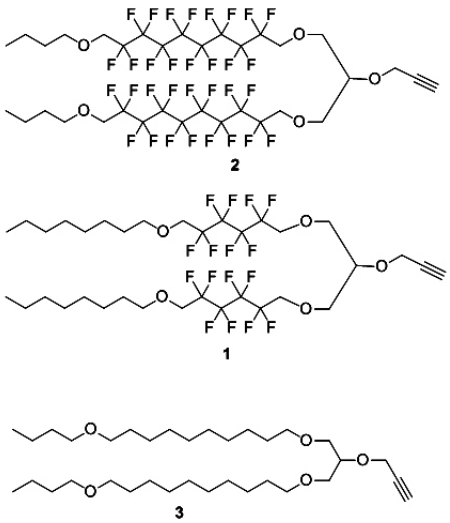
Copper wire was purchased from Aldrich (Steinheim, Germany). HPLC grade acetonitrile was obtained from Labscan (Bangkok, Thailand). All other reagents were obtained at the highest available purity from Sigma-Aldrich (Castle Hill, NSW, Australia). ESI-MS was performed using a Perkin-Elmer-Sciex API 3000 instrument with Analyst 1.4 software (Applied Biosystems/MDS Sciex, Toronto, Canada). Analytical RP-HPLC was performed using Shimadzu (Kyoto, Japan) instrumentation (DGU-20A5, LC-20AB, SIL-20ACHT, SPD-M10AVP) with a 1 mL min^{-1} flow rate and detection at 214 nm and/or evaporative light scattering detector (ELSD). Separation was achieved using a 0-100% linear gradient of solvent B over 40 min with 0.1% TFA/H_2O as solvent A and 90% MeCN/0.1% TFA/H_2O as solvent B on either a Vydac analytical C4 column (214TP54; 5 µm, 4.6 mm × 250 mm) or a Vydac analytical C18 column (218TP54; 5 µm, 4.6 mm × 250 mm). Preparative RP-HPLC was performed using Shimadzu (Kyoto, Japan) instrumentation (either LC-20AT, SIL-10A, CBM-20A, SPD-20AV, FRC-10A or LC-20AP x 2, CBM-20A, SPD-20A, FRC-10A) in linear gradient mode using a 5-20 mL/min flow rate with detection at 230 nm. Separations were performed with solvent A and solvent B on a Vydac preparative C18 column (218TP1022; 10 µm, 22 mm × 250 mm), Vydac semi-preparative C18 column (218TP510; 5 µm, 10 mm × 250 mm) or Vydac semi-preparative C4 column (214TP510; 5 µm, 10 mm × 250 mm).

Particle size was measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano Series with DTS software with non-invasive backscatter. Multiplicate measurements were performed at 25 °C with a scattering angle of 173° using disposable cuvettes and the number-average hydrodynamic particle diameters are reported.

Synthesis of Vaccine Candidate Lipopeptide *4*

A mixture of azide derivative *9* (2.6 mg, 0.6 µmol, 1 equiv.) and the lipoalkyne *1* (1.0 mg, 0.9 µmol, 1.5 equivalent) was dissolved in DMF (1 mL), and a copper wire (80 mg) was added. The reaction mixture was degassed for 30 sec by nitrogen bubbling, protected from light with aluminium foil, and stirred at 50 °C under nitrogen. The progress of the reaction was monitored by analytical HPLC (C-4 column) and ESI-MS until the peptide *9* was completely consumed after 5 h. The reaction mixture was purified by using semi-preparative HPLC C-4 column (35-75% solvent B over 60 min). After lyophilisation, the pure lipopeptide *4* was obtained as an amorphous white powder. Compound *4* was analysed by HPLC (C-4 column) t_R=27.9 min, purity >97% (detected by UV at 214 nm). Yield: (3.1 mg, 95%). ESI-MS: m/z 1657.9 (calc 1658.3) $[M+3H]^{3+}$; 1243.8 (calc 1243.7) $[M+4H]^{4+}$; 995.2 (calc 995.2) $[M+5H]^{5+}$; MW 4970.8.

Figure 1: Fluorinatedlipo-alkynes *1-2* and non-fluorinated lipo-alkyne *3*.



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Synthesis of Vaccine Candidate Lipopeptide *5*

A mixture of azide derivative *9* (3.1 mg, 0.7 µmol, 1 equiv.) and the lipoalkyne *2* (0.9 mg, 1.1 µmol, 1.6 equiv.) was dissolved in dimethylformamide (DMF) (1 mL) and a copper wire (80 mg) was added. The reaction mixture was degassed for 30 sec by nitrogen bubbling, protected from light with aluminium foil and stirred at 50 °C under nitrogen. The progress of reaction was monitored by analytical HPLC (C-4 column) and ESI-MS until the peptide *9* was completely consumed after 4 h. The reaction mixture was purified by using semi-preparative HPLC C-4 column (35-75% solvent B over 60 min). After lyophilisation, the pure lipopeptide *5* was obtained as an amorphous white powder. Compound *5* was analysed by HPLC (C-4 column) t_R=27.4 min, purity >97% (detected by UV at 214 nm). Yield: (3.6 mg, 98%).ESI-MS: m/z 1562.0 (calc 1562.0) $[M+3H]^{3+}$; 1171.9 (calc 1171.8) $[M+4H]^{4+}$; 937.7 (calc 937.6) $[M+5H]^{5+}$; MW 4683.0.

Biological Assay

Mice and cell lines for cellular immunity: Female C57BL/6 (6-8 weeks old) mice were used in this study and purchased from Animal Resources Centre (Perth, Western Australia). TC-1 cells (murine C57BL/6 lung epithelial cells transformed with HPV-16 E6/E7 and ras oncogenes) were obtained from TC Wu.[19] TC-1 cells were cultured and maintained at 37 °C/5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and 1% nonessential amino acid (Sigma-Aldrich). The animal experiments were approved by the University of Queensland Animal Ethics committee (DI/034/11/NHMRC) and (UQDI/327/13/NHMRC) in accordance with National Health and Medical research Council (NHMRC) of Australia guidelines.

***In vivo* tumor treatment experiments:** Five groups of C57BL/6 mice (5 per group) were challenged subcutaneously in the right flank with 1×10^5/mouse of TC-1 tumor cells. On the third day after tumor challenge, the mice were injected subcutaneously at the tail base with 100 µg of lipopeptide *4, 5*, or *6* in a total volume of 100 µL of sterile-filtered PBS. The positive control received 30 µg of a mixture of $E7_{44-57}$ and $E6_{43-57}$ emulsified in a total volume of 100 µL of Montanide ISA51 (Seppic,France)/PBS (1:1, v/v). A negative control group wasadministered 100 µL PBS. The mice were given a single immunisation only. The size of the tumor was measured by palpation and calipers every two days and reported as the average tumor size for the group of five mice or as the tumor size for individual mice.[20,21]

Tumor volume was calculated using the formula V $(cm^3)=3.14\times[\text{largest diameter}\times(\text{perpendicular diameter})^2]/6$.[21] To minimise suffering, the mice were euthanised when the tumor reached 1 cm^3 or started bleeding.

Statistical Analysis

All data were analysed using GraphPad Prism 5 software. Kaplan−Meier survival curves for tumor treatment experiments were applied. Differences in survival treatments were determined using the log-rank (Mantel-Cox) test, difference in tumor sizes were determined using two-way ANOVA; $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Human papillomavirus (HPV) oncoproteins E6 and E7 are continuously expressed and are essential for maintaining HPV-associated tumor cell growth. Peptides from the E6 and/or E7 proteins, $E6_{43-57}$ (QLLRREVYDFAFRDL; $E6_{43-57}$)[7,8] and $E7_{44-57}$ (QAEPDRAHYNIVTF; $E7_{44-57}$),[9] contain CTLs epitopes, and are therefore regularly used for the development of peptide-based vaccines against cervical cancer.[6,7,9-12]

We recently demonstrated that epitopes $E6_{43-57}$ and $E7_{44-57}$ conjugated to lipid *3* can stimulate potent cellular immune response and eradicate tumor in mice without the help of an external adjuvant.[6] Herein, we conjugated fluorinated analogues of

Figure 2: Synthesis of Vaccine Candidates *4-6*.

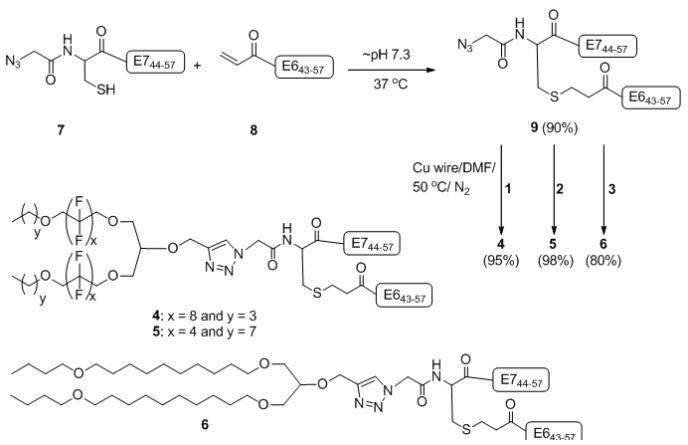

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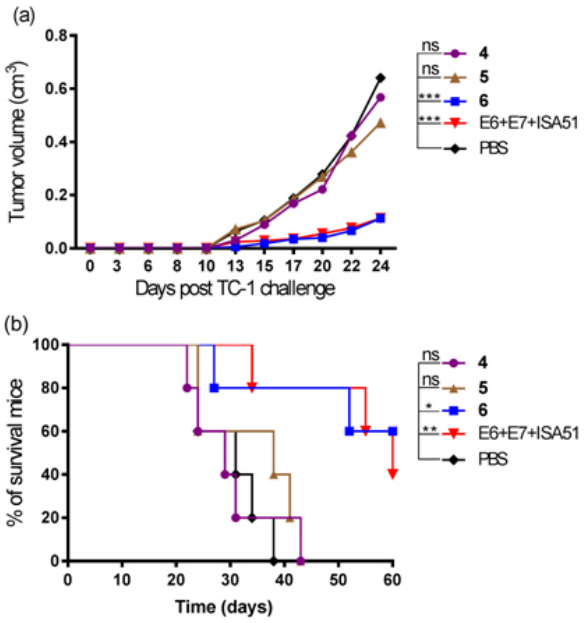
Figure 3: *In vivo* Tumor Treatment Experiments. C57BL/6 (5 per group) were Inoculated Subcutaneously in the Right Flank with 1×10^5 TC-1 Tumor Cells/Mouse (day 0) and Immunised with Lipopeptides *4, 5, 6*, a Mixture of $E7_{44\text{-}57}$-$E6_{43\text{-}57}$ (1:1) + ISA51 as a Positive Control, or PBS as a Negative Control on day 3. (a) Mean Tumor Volume (cm^3) in Different Groups of Mice Shown Up to Day 24 after Tumor Implantation (when the First Mouse was Euthanised). The Tumor Volume of each Group was Compared to the Negative Control (PBS) and was Analysed using Two-way ANOVA followed by Tukey's multiple Comparisons Test (*p<0.05; **p<0.01; ***p<0.001). (b) Survival rate Monitored over 60 days Post-implantation and time to Death plotted on a Kaplan-Meier Survival Curve. Mice were Euthanised when Tumor Volume Reached 1 cm^3 or Started Bleeding. The Survival Rate of each Group was Compared to the Negative Control (PBS) and was Analysed using the Log-Rank (Mantel-Cox) test (*p<0.05; **p<0.01).



this lipid, *1* and *2* (Figure 1) and covalently linked epitopes $E6_{43\text{-}57}$ and $E7_{44\text{-}57}$ (*9*) to produce lipopeptides *4* and *5* (Figure 2). At first, synthesis of $E7_{44\text{-}57}$ mercapto-azide (*7*) and $E6_{43\text{-}57}$ acrylate (*8*) was achieved by Fmoc solid phase peptide synthesis (Fmoc-SPPS). A double conjugation strategy[6] was used to synthesise lipopeptides *4-6*. The first conjugation of the two modified peptides *7* and *8* was accomplished though the Michael-addition mercapto-acrylate reaction to give the E6/E7 azide derivative *9* in 90% yield (Figure 2).[6] The final constructs *4-6* were achieved *via* the second conjugation between the azide derivative *9* and the lipo-alkynes *1-3* under nitrogen atmosphere using the Cu-AAC reaction in the presence of copper wire[13] at 50 °C (Figure 2).

Lipopeptides usually have amphiphilic properties and can self-assemble into particles under aqueous conditions.[14] Therefore, lipopeptides *4-6* were dissolved/suspended in PBS and vortexed or sonicated to form a homogenous solution. The particle sizes were measured by dynamic light scattering (DLS) with at least five repetitions. Compound *6* formed 450-750 nm particles while compounds *4* and *5* aggregated toform larger particles with sizes above the detection level of the instrument (>5 µm). The fluorinated compounds *4* and *5* formed milky suspension.

The ability of the fluorinated multi-antigenic conjugates *4-6* to eradicate tumor cells was evaluated *in vivo* in a C57BL/6 mouse model of HPV-positive tumors. At day zero, mice (5 mice/group) were implanted in the side flank with E6/E7 positive tumor cells.[15] On day three,mice were immunised with either lipopeptide *4, 5, 6*, a mixture of $E7_{44\text{-}57}$+$E6_{43\text{-}57}$ emulsified with incomplete Freund's adjuvant (Montanide ISA51) as a positive control, or PBS as a negative control. Unfortunately, persistent tumor growth was observed, thus fluorinated lipopeptides *4* and *5* had a negligible therapeutic effect against the tumor (Figure 3). In contrast, mice immunised with non-fluorinated analogue (*6*)[6] displayed a 60% survival rate (3 out of 5 mice).

The availability of non-toxic and efficient adjuvants that can stimulate cellular and/or humoral immunity without causing side effects is very limited.[3] Peptide alone is unable to stimulate the required immune response, thus there is increased demand for the discovery of new molecules that can play this crucial adjuvanting role. Here, we synthesized fluorinated and non-fluorinated lipopeptides *4-6* (Figure 2). The stimulation of cellular immunity was tested by comparing fluorinated (*4-5*) and non-fluorinated (*6*) vaccine candidates that bore two HPV-16 epitopes derived from E6 and E7 (Figure 3). The hydrophobic properties of the two epitopes $E6_{43\text{-}57}$ and $E7_{44\text{-}57}$ contributed to

the formation of big particles of compounds *4-6* in PBS. In addition, fluorine atoms led to a reduction in the solubility of the fluorinated derivatives *4* and *5* and resulted in aggregated particles that were too large to measure by DLS. The non-fluorinated analogue *6* resulted in smaller particles (450-750 nm). The large particle size of fluorinated compounds *4* and *5* (with E6/E7 epitopes >5 μm) could be the reason for their poor immunological potency (Figure 3). In large aggregates of *4* and *5*, the antigen might be hidden from the immune system. This was supported by recent experiments where aggregated compounds that contained Pam2Cys-E6/E7also (>5 μm) failed to elicit an immune response that stopped tumor growth.[6] Similarly, the large microparticles of the poly *tert*-butyl acrylate-E6/E7 conjugate had a limited efficacy in tumor challenge experiments in mice (10% survival rate) while acrylate conjugates that formed smaller particles were significantly more effective (50% survival rate).[16] It was also reported that large particles of ovalbumin loaded-beads failed to induce a strong IFN-γ response, a measure of potent cellular immune activation.[17,18] The particle size might be the reason why the same fluorinated lipids, when formed small nanoparticles upon conjugation with a highly hydrophilic B-cell epitope, induced a strong humoral response[5]; while when they formed big microparticles in conjugation with the hydrophobic E6/E7 epitopes, they showed a weak cellular response. In our previous work, non-fluorinated analogue *6* was able to produce $CD8^+$ T-cell response[6]; however, as fluorinated lipopeptides *4* and *5* did not induce promising antitumor immune responses, we did not perform further cellular immunity studies on them. The size of self-assembled fluorinated lipopeptides can be reduced in the future through the introduction of a hydrophilic spacer moiety, such as polyethylene glycol, between fluorinated lipid and peptide epitopes.

CONCLUSION AND FUTURE PROSPECT

Three multi-antigenic lipopeptide vaccine candidates *4-6* were synthesised using a double conjugation technique. The non-fluorinated multi-antigenic lipopeptide analogue *6* stimulated a robust therapeutic effect against HPV- positive tumors after a single immunisation without the help of an external adjuvant. The fluorinated self-adjuvanting moieties (*4* and *5*) were not able to eradicate tumor, possibly due to the spontaneous formation of large (>10μm) aggregates in aqueous solution. Therefore further study is required to examine whether it is possible to form small nanoparticles from T-cell epitopes conjugated to fluorinated lipids.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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Review

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Immunobiology of Anticancer Virotherapy With Newcastle Disease Virus in Cancer Patients

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ABSTRACT

Virotherapy with oncolytic viruses that preferentially infect and kill cancer cells is a novel and promising strategy for cancer treatment. Newcastle disease virus (NDV), which is pathogenic in birds, has beneficial clinical effects in cancer patients. NDV virotherapy is safe and elicits an antitumor response in patients affected by different types of cancers. The selective replication of NDV in tumor cells, the lack of genetic recombination, the lack of interaction with host cell DNA, and safety of NDV vaccination in cancer patients has resulted in NDV virotherapy to be accepted as a potentially attractive anticancer modality. However, more knowledge is needed to support the development of optimal NDV-based treatment modality for cancer. In this paper, the biological characteristics of NDV, the clinical effectiveness of NDV-based anticancer vaccination, immunobiology of NDV virotherapy in cancer patients, immune responses to NDV vaccines, and NDV-induced immunogenic cell death and apoptosis of cancer cells have been discussed in detail.

KEY WORDS: Virotherapy; Newcastle disease virus (NDV); Cancer treatment; Antitumor immune responses; Apoptosis.

ABBREVIATIONS: NDV: Newcastle Disease Virus; nm: Nanometer; NP: Nucleoprotein; P: Phosphoprotein; M: Matrix Protein; F: Fusion Protein; HN: Hemagglutinin-Neuraminidase; L: Large Protein; IFN: Interferon; RIG-1: Retinoic Acid-Inducible Gene 1; BCG: Bacillus Calmette-Guerin; DTH: Delayed Type Hypersensitivity; IL: Interleukin; PSA: Prostate-Specific Antigen; NK cell: Natural Killer cell, CTLA-4: Cytotoxic T-Lymphocyte Associated Antigen 4; PAMPs: Pathogen-Associated Molecular Patterns; PPRs: Pattern Recognition Receptors; dsRNA: Double Stranded RNA; PKR: Protein Kinase R; TLRs: Toll Like Receptors; NO: Nitric Oxide; TRAIL: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand; TNF: Tumor Necrosis Factor; IFNRA: Interferon Receptor Alpha; DC1: Type I Dendritic Cell; Th1: Helper 1 T-Cell; ELISPOT: Enzyme Linked ImmunoSpot.

INTRODUCTION

Conventional cancer therapy modalities, including surgery, chemotherapy, and radiotherapy, do not have sufficient clinical efficacy in the treatment of advanced cancers and introduction of more effective therapeutic approaches is essential for treating patients with advanced forms of cancer. Virotherapy with oncolytic viruses that preferentially infect and kill cancer cells is a promising therapeutic strategy for cancer treatment. Several viruses, including vaccinia virus, herpes simplex virus, measles, adenovirus, vesicular stomatitis virus, myxoma virus, reovirus, lentivirus, and Newcastle disease virus (NDV) have been identified as oncolytic viruses in preclinical and clinical studies.[1-3] Virotherapy approaches have the potential to be employed as monotherapy or be used in combination with conventional cancer therapy modalities to improve the overall chances of the patient's survival and increase the percentage of treated patients with long-term survival. Further investigation has shown that NDV may be a suitable oncolytic agent for virotherapy of cancers.

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Anticancer properties of NDV have been intensively studied in the decades 1950s and 1960s.[4-6] Post-operative vaccination of mice with irradiated autologous tumor cells infected with NDV resulted in the disappearance of micrometastases from visceral organs, increased the survival of vaccinated mice, and helped cure the cancer in about 50% of the treated mice.[7] Favorable properties of NDV, including selective replication of NDV in tumor cells, lack of genetic recombination, lack of interaction with the host cell DNA, and safety of NDV vaccination in cancer patients, led to the clinical application of NDV virotherapy as an anticancer treatment of choice. In several clinical trials, NDV virotherapy has been medically implemented in patients with different types of cancer such as colorectal carcinoma, melanoma, renal cell carcinoma, breast cancer, ovarian cancer, glioblastoma multiform, head and neck squamous cell carcinoma, and prostate cancer. This virotherapy approach was considered as clinically safe and could help support antitumor effects in patients with advanced forms of cancer.[3]

BIOLOGICAL CHARACTERISTICS OF NDV

Biology of NDV

NDV, with a spherical shape, 150 nm diameter, and a lipid bilayer envelope, belongs to the genus *Avulavirus* in the family *Paramyxoviridae*. This virus has a single-stranded, negative-sense, nonsegmented RNA genome of approximately 15,186 nucleotides, which contains six genes, including nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L). These genes encode at least seven proteins. NDV harbors a single-stranded RNA-dependent RNA polymerase complex that consists of the L, P, and NP proteins.[8-10] The V protein, which is encoded by the P gene through an overlapping reading frame, functions as an IFN type 1 antagonist in avian hosts.[11,12] NDV is an RNA virus and it replicates in the cytoplasm of infected cells without a DNA stage, thus, the possibility for genetic recombination with host cell DNA is very rare.[13]

Pathogenic Classification of NDV

NDV is an animal pathogen which infects various avian species. Different strains of NDV causes a contagious viral disease in most domestic and wild avian species. NDV strains are classified into three pathotypes based on their virulence in birds, classified as velogenic, mesogenic, and lentogenic strains. Velogenic strains are one of the most commonly observed pathogenic NDV strains, responsible for causing a severe infection resulting in a high incidence of mortality in infected chickens. The common signs of ND include depression, fever, loss of appetite, abnormal thirst, severe dehydration, and emaciation. The mortality rate can reach up to 100% on account of this condition. Mesogenic strains are mid-virulent NDV strains, causing respiratory disease in chicks and young chickens and reducing their egg-laying ability. These strains may result in up to 25% mortality. Lentogenic strains are non-virulent attenuated strains, causing mild symptoms in the respiratory tract of infected birds.[10,14] In humans, NDV alone can cause transient conjunctivitis and mild flu-like symptoms and poses no hazard to the human health. So far, several mesogenic and lentogenic strains of NDV have been successfully used in oncolytic virotherapy in mouse tumor models and cancer patients, such as *PV701* (strain *73-T*), *LaSota*, and *Ulster*.[3] NDV strains can also be categorised into lytic and nonlytic strains. Both oncolytic and non-oncolytic NDV strains have been used in the clinical treatment of patients affected with cancer.

Selective Replication of NDV in Human Cancer Cells

NDV replicates in most human cancer cells and destroys various types of cancer cells such as fibrosarcoma, osteosarcoma, cervical carcinoma, bladder carcinoma, neuroblastoma, pancreatic adenocarcinoma, pleural mesothelioma, and Wilm's tumor cell lines, both *in vitro* and *in vivo*.[15-17] It has been observed that the virus yield increases 10,000-fold within 24 hours in the tumor and chick embryo cells supernatant, but the titer values remains near zero in the normal fibroblast supernatant.[15] Human pancreatic tumor cell lines also show more than 700 times higher sensitivity than normal cells to the NDV killing *in vitro*.[16] Moreover, NDV is a biological agent with a potential to disrupt the resistance of cancer cells to therapy on account of its ability to replicate in non-proliferating tumor cells which are resistant to chemotherapy and radiotherapy.[18] NDV can also replicate in hypoxic cancer cells.[19]

Mechanisms Involved in the Selective Replication of NDV in Cancer Cells

The molecular mechanisms underlying the NDV-sensitivity of human cancer cells have been investigated in some studies. Selective replication of NDV in tumor cells is suggested to be associated with defects in the antiviral defense mechanisms in tumor cells. Decreased IFN expression and impaired induction of IFN-induced antiviral proteins in tumor cells have been shown to be correlated with efficient NDV replication.[20-22] But, there are some existing evidences that indicate that other mechanisms are also involved in the selective replication of NDV in human tumor cells. Some strains of NDV with intact IFN-antagonistic function, containing V protein, can replicate in normal human cells. In a multistage skin carcinogenesis model derived from nontumorigenic HaCaT cells, there was no significant difference in interferon signaling between virus-sensitive tumor cells and virus-resistant nontumorigenic parental cells. In this epithelial cancer cell line model, Rac1, a pleiotropic regulator of multiple cellular functions, was considered as an oncogenic protein that is essential for NDV replication in tumorigenic cells. Additionally, Rac1 expression was sufficient to render nontumorigenic cells susceptible to NDV replication and to oncolytic cytotoxicity.[23] In a nude mouse model of human fibrosarcoma, IFN-sensitive recombinant NDV was as effective as IFN-resistant virus in the elimination of tumor burden.[24] No correlation was observed

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between defects in IFN pathways and NDV replication or NDV-induced cytotoxicity in 11 different human pancreatic adenocarcinoma cell lines. Pretreatment of cell lines with IFN resulted in diminished NDV replication and its cytotoxic effects in most cell lines.[25] Tumor selectivity of NDV has also been dependent on the expression of retinoic acid-inducible gene 1 (RIG-1), a cytosolic RNA sensor.[26] As a consequence, several mechanisms are associated with the selective replication of NDV in tumor cells such as defects in the activation of antiviral defense pathways especially type I interferon signaling pathways,[20-22] activation of Ras signaling and expression of Rac1 protein,[23] as well as defects in apoptotic pathways.[27]

ANTICANCER EFFECTIVENESS OF NDV-BASED VACCINATION IN CLINICAL TRIALS

To date, four NDV-based vaccination approaches have been implemented in clinical trials, including vaccination with free infectious NDV, vaccination with intact, irradiated, tumor cells infected in culture by NDV, vaccination with lysate from NDV-infected tumor cells, and vaccination with *ex vivo* generated dendritic cells pulsed with lysate from NDV-infected tumor cells.[3] In clinical trials of patients with solid cancers, administration of NDV particles resulted in some clinical responses. General virus induced-side effects were flu-like symptoms, tumor site-specific adverse events, and infusion reactions.[28-32] In a ten-year follow-up of stage II malignant melanoma patients treated postsurgically with NDV oncolysate (tumor cell lysate), post-operative vaccination with NDV oncolysate was able to improve the survival of stage II malignant melanoma patients.[33] In contrast, post-operative vaccinations with lysate from autologous melanoma cells infected with NDV *Ulster* strain in combination with administration of IL-2 did not show clinical efficacy in melanoma patients with resectable stage III disease.[34] In other clinical trials, vaccination with NDV-infected autologous tumor cells elicited clinical responses and increased the survival rate of patients particularly affected by colorectal cancer, renal cell carcinoma, breast cancer, ovarian cancer, glioblastoma multiform, and head and neck squamous cell carcinoma.[3] In general, vaccination with NDV-infected autologous tumor cell vaccines have showed greater therapeutic efficacy than vaccination with NDV particle vaccines and NDV-infected tumor cell lysate vaccines.[3]

In colorectal patients vaccinated post-operatively with autologous tumor cell vaccine and NDV vaccine, survival rates were more than that in patients treated with surgery plus radiotherapy or chemotherapy.[31] In addition, vaccination with NDV-infected autologous colorectal tumor cells was more effective than vaccination with tumor cells admixed with bacillus Calmette-Guerin (BCG) in eliciting antitumor responses in resected colorectal carcinoma patients. Also, NDV-infected tumor cell vaccines induced mild side effects while vaccination with BCG-admixed tumor cells led to the development of long-lasting ulcers and serious side effects.[35] Nevertheless, vaccination with NDV-infected autologous tumor cells did not improve the overall survival of stage IV rectal cancer patients following resection of liver metastasis when compared with non-vaccinated patients for a follow-up period of about 10 years.[36] Recent resources have shown that vaccination with dendritic cells pulsed with lysate from NDV-infected autologous tumor cells in cancer patients resulted in cancer regression.[37-38]

IMMUNOBIOLOGY OF NDV VIROTHERAPY IN CANCER PATIENTS

In a phase I/II trial in patients with recurrent glioblastoma multiform, anti-NDV hemagglutinin antibodies were detected following the administration of intravenous injections of NDV and viral particles which were recovered from the blood, saliva, urine samples, and one tumor biopsy.[39] However, neutralizing antibodies generated during NDV treatment may interfere with the antitumor effectiveness of NDV vaccines. In a clinical trial involving colorectal patients vaccinated post-operatively with autologous tumor cell vaccine and NDV vaccine, there was an association established between skin delayed type hypersensitivity (DTH) reaction and the prognosis of treated patients.[31] In other clinical trials involving patients of colorectal cancer with liver metastases, vaccination with NDV-infected, irradiated, autologous tumor cells following curative liver resection resulted in an increased sensitization against autologous tumor cells, as measured by DTH reactivity. Importantly, a strong correlation between increased skin DTH reaction against autologous tumor cells and recurrence-free interval was observed in the vaccinated patients.[40] Postsurgical vaccination of colorectal cancer patients with NDV-infected autologous tumor cell vaccine was also associated with increased skin DTH reactivity and a dense infiltration of predominantly helper T-cells in the vaccination site.[41]

In patients with glioblastoma multiform postsurgically vaccinated with NDV-infected autologous tumor cells, a significant increase in the skin antitumor DTH reactivity, improved survival, and increased numbers of tumor reactive memory T-cells in the peripheral blood and $CD8^+$ tumor infiltrating T-cells were observed in the secondary tumors of vaccinated patients.[42] Significant increase in the antitumor skin DTH reactivity and the presence of tumor reactive T-cells in the peripheral blood, even 5 to 7 years after vaccination, were observed in a significant proportion of head and neck squamous cell carcinoma patients vaccinated postsurgically with NDV-infected autologous tumor cells.[43] Preconditioning of head and neck squamous cell carcinoma patients with IL-2 prior to vaccination was associated with an increase in the number of T-cells and augmented antitumor DTH reactivity.[44] In patients with advanced renal cell carcinoma with distant metastases, multiple vaccinations with NDV-infected autologous tumor cells after nephrectomy followed by administration of low doses of IL-2 and IFN-α resulted in a complete response in 12.5% and partial response in 15% of the vaccinated patients.[45] Genetic manipulation of NDV towards arming the virus with genes encoding cytokines or tumoricidal molecules is also being investigated to improve

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the antitumor effects of NDV-based vaccines.[46-50]

In a patient with hormone-refractory metastatic prostate cancer who had failed to cope with standard cancer therapy, postsurgically intravenous administration of NDV in combination with vaccination with autologous monocyte-derived dendritic cells pulsed with NDV-infected tumor cell lysate and administration of IFN-γ, resulted in complete remission of prostate cancer, long-lasting dramatic decrease in prostate-specific antigen (PSA) levels, induction of antitumor memory T-cell response, and a reduction in bone metastases.[51] Similarly, long-term survival of another patient with invasive ductal breast cancer and primary liver metastases was observed upon the postsurgical application of radiofrequency for treating hyperthermia of the liver, intravenous administration of NDV, and vaccinations with autologous monocyte-derived DCs pulsed with lysate from NDV-infected breast cancer cells. Sustained tumor-specific memory T-cell response was observed upon the administration of dendritic cell vaccinations.[52]

Induction of immunogenic cell death as well as induction of apoptosis in cancer cells were involved in the NDV-mediated killing of cancer cells upon NDV vaccination in affected patients.

INDUCTION OF ANTITUMOR IMMUNE RESPONSES TO NDV VACCINATION AND IMMUNOGENIC CELL DEATH OF CANCER CELLS

Virus-induced stimulation of different immune cells can be responsible for strong antitumor responses of NDV in tumor-bearing hosts.[53] The prevention of metastatic spread by postsurgical vaccination with NDV has been paralleled with an establishment of specific systemic antitumor immunity.[54] Presentation of NDV-encoded antigens on the cell surface of infected cancer cells induces the stimulation of lymphocytes. Two of six NDV genes, HN and F, modify the tumor cell surface which leads to enhanced lymphocyte interactions. Other viral genes can also stimulate a number of host cell genes leading to the production of several cytokines and chemokines. Furthermore, double-stranded RNA produced in NDV-infected cells activates antiviral immune responses based on type I interferons such as IFN-α and IFN-β. Nonetheless, NDV selectively replicates in murine/human tumor cells as the V protein, which inhibits type I interferon responses in permissive NDV-infected avian cells, which does not interfere with the interferon response in mammalian cells.[11,12]

NDV has a capability to co-stimulate tumor-specific cytotoxic T-lymphocytes.[55,56] Tumor-specific cytotoxic T-cell response observed in mice immunized with NDV-infected tumor cells was mediated using IFN-α/β.[56] NDV infection of melanoma cell line completely restored the proliferative response of tumor tissue-derived $CD4^+$ T-cell clone and inhibited the induction of T-cell anergy to melanoma by the induction of B7-1/B7-2-independent T-cell costimulatory activity in human melanoma cells.[57] It has been found that NDV-infected tumor cells enhance tumor-specific T-cell responses as a result of $CD4^+$ and $CD8^+$ T-cell cooperation.[58] NDV-infected tumor cell vaccine augmented tumor-specific cytotoxic $CD8^+$ T-cell responses and $CD4^+$ T helper activity in a mouse lymphoma model.[59] NDV induced long-term survival and tumor specific T-cell memory through induction of immunogenic cell death in an orthotopic glioma model.[60]

NDV antigens expressed on antigen presenting cells or tumor cells can augment peptide-specific T-cell responses.[61] NDV-derived HN molecules facilitated adhesive interactions of lymphocytes with NDV-infected tumor cells.[62] Vaccination of late-stage metastasized colorectal carcinoma patients with NDV-infected tumor cells attached with NDV-specific single chain antibodies with specificity for the HN and CD28 induced tumor-specific T-cells in all vaccinated patients, and 28.6% of patients showed a partial response.[63] HN protein can activate natural killer (NK) cells. In a mouse tumor model, vaccination with a plasmid encoding the HN protein of NDV resulted in a significant increase in NK cell infiltration and a decrease in infiltration of myeloid-derived suppressor cells.[64] Combinational therapy with localized NDV and systemic anti-CTLA-4 blockade led to rejection of pre-established tumors and protection from tumor rechallenge in poorly immunogenic tumor models, melanoma (B16 cells) and colon cancer (MC38 cells). This combinational therapy resulted in distant tumor infiltration with $CD4^+$ and $CD8^+$ T cells and its therapeutic efficacy was dependent on the $CD8^+$ T cells, NK cells, and type I interferon.[65]

Intratumoral injection of NDV in athymic mice resulted in complete regression of human fibrosarcoma and neuroblastoma xenografts,[66] indicating that other immune cells, other than T-cells, are involved in the NDV-induced antitumor immune responses. Pathogen-associated molecular patterns (PAMPs) of NDV can be recognized by pattern recognition receptors (PPRs) of innate immune cells, including cytoplasmic RIG-1, cytoplasmic dsRNA dependent protein kinase R (PKR), endosomal Toll-like receptors (TLRs), plasma membrane expressed NK cell receptor NKp46, leading to initiation of multiple signaling pathways, and subsequently, strong type I interferon response, release of proinflammatoy cytokines, and activation of other immune cells.[67]

NDV can activate macrophages. NDV induces nitric oxide (NO) synthesis in infected macrophages *via* activation of nuclear factor-kappa B.[68] NDV also stimulates tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated tumoricidal activity of human monocytes.[69] HN protein of NDV induces cell surface expression of TRAIL and secretion of IFN-α in human peripheral blood mononuclear cells.[70] NDV-activated murine macrophages upregulated antitumor molecules NO and TNF-α, and showed antitumor cytostasis and cytotoxicity *in vitro*. The antitumor cytotoxicity of NDV-activated macrophages was used against various tumor cell lines. Intravenous transfer of NDV-activated macrophages resulted in a significant

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suppressive effect on pulmonary metastases in mammary carcinoma and lung carcinoma models.[71]

NDV can also activate dendritic cells. Viral RNA in the NDV oncolysate pulsed dendritic cells acts as a PAMP. Recognition of viral RNA in NDV-infected cells by endosomal TLRs, such as TLR-3, -7, and -8, and the cytoplasmic retinoic acid inducible gene1 (RIG-I) induces a strong type I interferon response.[72] IFN-α and IFN-β molecules secreted by NDV-infected cells interact with the cell surface type I interferon receptor (IFNRA) and initiate intracellular signaling pathways leading to the blockage of viral replication in the target cells.[73] *In vitro* stimulation of human monocyte-derived dendritic cells with NDV polarized dendritic cells towards type I dendritic cells (DC1) induce helper 1 T-cell (Th1) responses.[74] NDV oncolysate-pulsed dendritic cells potently stimulated autologous T-cells in breast cancer patients. They increased the expression level of costimulatory molecules in comparison to tumor lysate-pulsed dendritic cells and elicited greater IFN-γ ELISPOT responses. Supernatant from cocultures of NDV oncolysate-infected dendritic cells and bone marrow cancer reactive T-cells contained increased titers of IFN-α and IL-15.[75]

Recently, an NDV oncolysate-pulsed dendritic cell vaccine has been clinically administered to patients at the Immunological and Oncological Center (IOZK) in Cologne, Germany.[37,38] Before receiving the vaccination, patients were preconditioned by electrohyperthermia to activate the immune system and to enhance the virus tumor targeting and replication. It is possible that induction of NDV oncolysate-specific T-cells help recall T-cell responses upon dendritic cell vaccination and augment the generation of effective antitumor T-cell responses.[38]

NDV-MEDIATED INDUCTION OF APOPTOSIS IN CANCER CELLS

In addition to immunogenic cell death, induction of apoptosis appeared to be an important mechanism of NDV-mediated cancer cell killing. Intratumoral injection of recombinant NDV strains derived from the velogenic strain *Italien* induced syncytium formation and cell death as well as prolonged survival of the tumor-bearing mice.[76] Human tumor cell infection by NDV leads to upregulation of MHC and cell adhesion molecules, induction of interferons, chemokines and finally apoptosis.[77] Also, velogenic NDV AF2240 strain has been reported to induce apoptosis in a time-dependent manner on the mammary carcinoma cell line.[78]

In both the intrinsic and extrinsic pathways of apoptosis, caspases, cysteine aspartyl-specific proteases that cleave structural cytoplasmic and nuclear proteins, are activated, leading to the biochemical and morphological changes. Recombinant NDVs have mediated cytotoxicity against human tumor cell lines by inducing apoptosis through multiple caspase-dependent and IFN-independent pathways. NDV primarily triggered apoptosis by the activation of the intrinsic mitochondrial death pathway. Early activation of caspase-9 and effector caspase-3 was detected in NDV-infected tumor cells as early as 6-8 hours, indicating that intrinsic apoptotic pathways operate early in NDV-infected tumor cells. Activation of caspase-8 was detected in many of the tumor cell lines 48 hours after the NDV infection of cells but it was dispensable for inducing apoptosis. Cleavage of caspase-8, which is predominantly activated by the death receptor pathway, was a TRAIL-induced late event. Moreover, caspase-8 and caspase-9 inhibitors suppressed biochemical and morphological changes of the NDV-infected tumor cells. But, caspase-8 and caspase-9 inhibitors did not completely abrogate the signs of apoptosis in NDV-infected tumor cells. In addition, caspase inhibitors had no effects on virus replication.[79] Releasing multiple tumor antigens upon lysis of NDV-infected tumor cells is also responsible for inducing immune-mediated antitumor therapeutic response.

CONCLUSION

Various NDV strains selectively replicate in and kill human tumor cells. NDV-based vaccinations have helped increase the survival rate of cancer patients in several clinical studies. NDV vaccination in cancer patients can activate different immune cells with antitumor activity. Immunogenic cell death and induction of apoptosis are involved in the NDV-mediated killing of cancer cells. Interestingly, NDV virotherapy can be combined with other anticancer modalities, such as surgery, chemotherapy, and diverse immunotherapy approaches to induce stronger antitumor responses and eradicate residual tumor cells which persist following conventional therapy. Genetic manipulation of NDV to express genes encoding cytokines and other immunostimulatory molecules, and identifying NDV strains with potential antitumor effects are presently being investigated to improve the antitumor efficacy of NDV-based vaccines.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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